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Nitrification and the nitrifying organisms Part I, Nitrifying organisms Part II, Certain conditions affecting the oxidation of ammonia and nitrite nitrogen Part III, Nitrates and nitrification in field soils

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NITRIFICATION AND THE NITRIFYING
ORGANISMS

PART I. NITRIFYING ORGANISMS

PART II. CERTAIN CONDITIONS AFFECTING THE OXIDATION
OF AMMONIA AND NITRITE NITROGEN.

PART III. NITRATES AND NITRIFICATION IN FIELD SOILS
by

Rayadurg Nagan Gowda.

A Thesis Submitted to the Graduate Faculty

for the Degree of

Doctor of Philosophy

Major subject: Soil Bacteriology.

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Graduate Dean

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1923

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NITRIFICATION AND THE NITRIFYING ORGANISMS.

PART I.

Introduction.

One of the most important elements in plant nutrition is nitrogen. It is taken in by most plants only in the form of nitrates. As far as is known at present nitrates are produced in soils only by the action of microorganisms. These organisms were only found after many investigators had conducted a long and tedious search. It was not until Winogradsky in 1890 (44) announced the isolation of two organisms that produce nitrates from ammonia that the search was successful. Ever since that time numerous workers have attempted to repeat Winogradsky's work. A few have claimed the isolation of organisms that form nitrites and nitrates and also conform to the requirement of purity of the cultures laid down by Winogradsky. This requirement was that they should not grow in alkaline bouillon. None of these investigators, however, have been successful in duplicating the work of Winogradsky in its entirety. The majority of investigators on the other hand have failed absolutely to secure cultures of organisms which would meet Winogradsky's criterion of purity, altho the methods of isolation followed by these workers have been very much the same as those of Winogradsky.

From this it must be inferred that either the nitrifying organisms will not grow in bouillon and the methods developed so far to separate them from those that grow in bouillon are unsatis-

factory to the extent that they cannot be successfully followed by other workers or that the nitrifiers grow in bouillon.

In view of the fact that the nitrifying organisms function in soil in the presence of large amounts of organic matter and that it has been shown (14, 24, 40) that some organic matter has a beneficial effect, the question arises whether growth or lack of growth in bouillon can be used as the criterion for determining the purity of cultures of nitrifiers.

The whole problem of isolation of the nitrifiers and the methods of such isolation therefore still remains to be solved. Schloesing and Muntz (35) were the first to show that the oxidation of ammonia to nitrite was due to microorganisms. The Franklands (12) obtained in a liquid medium a pure culture of a ammonia oxidizing organism. They described this organism as a "bacillo-coccus" about 0.8 μ in length and a little less in width. It would not grow in gelatine peptone solution or on any solid media. About the same time Warington (42) isolated two organisms, one oxidizing ammonia to nitrous acid and the other oxidizing nitrous acid to nitric acid. Neither of these organisms would grow on any solid media known then, or on any liquid media containing organic matter. In 1890 Jordan and Richards (21) also isolated an organism, or a group of organisms that oxidized ammonia to nitrate. Like the organisms of Frankland and Warington this group would not grow on any known solid media. It was obtained only in liquid cultures that contained no organic matter.

Winogradsky (44, 45, 46) solved the problem of growing

Nitrifying organisms on solid media by using the neutral inorganic silicic acid gel as substratum. He obtained these organisms by inoculating soil into a solution of ammonium sulphate supplemented by inorganic nutrient salts. When the greater part of the ammonia was oxidized to nitrite, he made transfers to fresh sterile solutions. This process later known as "sub-culture transfers" was repeated a great many times until most of the contaminating forms were eliminated by the simple process of making conditions favorable for the desired organisms.

He inoculated these liquid cultures into gelatine plates and after a suitable period of incubation picked out chunks of the medium that showed no visible growth. The chunks introduced into a sterile liquid medium produced vigorous oxidation, thus a majority of the contaminating forms are eliminated. This process is often termed the "negative plate" method. Later Winogradsky inoculated the liquid culture into silicic acid gel and obtained colonies supposed by him to be pure cultures.

He describes these colonies as being too small to be seen by the naked eye. Under enlargement of 100 they were refractive and with a clear outline, first colorless, later brown and finally dark brown in color. The organism is an oval or ellipsoidal cell 1.2μ to 1.8μ in length and 0.9μ to 1.0μ in width. It appears in two forms, one remaining in single cells and the other showing a zooglear-like growth. In liquid cultures the single cell state causes turbidity and possesses a single polar flagellum; in the zooglear state it does not cause

any turbidity. He gave this organism the name of Nitrosomonas.

A year later Winogradsky repeated the above experiments with nitrite solutions and obtained an organism to which he gave the name Nitrobacter, the organism causing oxidation of nitrites to nitrates. This organism was 1.0μ long and 0.3μ to 0.4μ wide, and occurred singly or in pairs and occasionally in threes.

He also used as substratum agar washed free of all soluble material and isolated pure cultures of the above organisms.

Omeliansky (29) reported on the various methods of isolating the nitrifiers. He tried the dilution method, the negative-plate method, silicic acid gel and washed agar plate methods. He rejected the first two as inadequate (methodischer Hinsicht) from the viewpoint of method. The washed agar method he considered inferior to the silicic acid gel method in that in the former the nitrifying organisms grow exceedingly slowly. He worked out a method (30) for using gypsum blocks as substratum for isolating the nitrifiers. He mixed calcium sulphate ($\text{CaSO}_4 \cdot \text{H}_2\text{O}$) containing 1 percent magnesium carbonate with enough water to make a dough and spread it in thin smooth plates. When dried he put them in petri dishes and sterilized. The nutrient solution containing the required salts was added taking care to keep the surface of the block moist without flooding it. The surface of the block was inoculated with the desired culture. The colonies were yellow and wart-like.

Three years later he (31) used filter paper pads as

substratum for growing nitrifiers with satisfactory results. Beddies (3) used a medium containing 1/4 percent water glass (wasserglaslösung), 1 percent of a concentrated humus solution and isolated nitrifying organisms. These he found were not very sensitive to high concentrations of organic matter; on the other hand they were helped by the organic matter used by him. Spore formation under certain conditions was observed.

Stutzer and Hartleb (40) isolated a nitrite oxidizing organism which they called Nitromicrobium which measured 1 to 1.2 μ in length and 0.75 to 1.0 μ in width. It multiplied by yeast-like budding. It did not differ from Winogradsky's Nitrobacter in other respects. In media containing nitrogen in the form of peptone, meat extract or asparagin no growth was obtained. Washed agar in soil extract was used as a solid medium. They also reported the isolation of a contaminating form which they called Hypomicrobium. This organism showed true branching and did not grow in media containing organic matter. It did not oxidize ammonia or nitrite.

The same year Stutzer (39) reported the isolation of Nitrosomonas by negative-plate method with silicic acid gel. On agar when observed under a magnification of 100 - 150 very small, sharp edged, almost round colonies were seen. The cells were not perfectly round or equal in size and measured 1 to 1.25 μ long and 0.7 to 1.0 μ broad. These did not grow on media containing organic matter.

By using silicic acid gel Boullanger and Massol (9) isolated the nitrifying organisms. Details of isolation and purity

are not found in their report.

Wimmer (43) reported the isolation of Nitrosomonas and Nitrobacter, but does not describe his methods. His Nitrobacter cultures gave growth in bouillon in his later experiments.

Perroti (32) recommends for the cultivation of nitrite formers magnesium carbonate blocks dipped in nutrient solution as suitable substratum. This paper gives a review of literature on the cultivation of nitrifying organisms.

Perroti (33) also isolated the organism causing the oxidation of ammonia. He described it as a coccus or a short rod measuring 0.6 to 0.8 μ with a cilium 1 μ long. These organisms appear in single cells and in chains of two to six cells. It does not form zoogles or grow in media containing organic matter. The growth of these organisms is accelerated by aeration, temperature of 20° C. and a nutrient solution of the right dilution.

Bazarewski (2) isolated nitrate forming organisms. Coleman (11) isolated both the organisms causing nitrification, but the Nitrosomonas was not obtain^{ed} in pure cultures.

Makrinoff (24) isolated Nitrosomonas by using gypsum and magnesium carbonate plates and in making these plates he added soil extract, dried and somewhat decomposed leaves, and soil rich in humus, and showed that the plates containing organic matter exerted a favorable influence on the oxidation of nitrites.

Beijerinck (4) isolated Nitrobacter on silicic acid gel and washed agar. The organism grew in bouillon profusely, but thereby lost its property of oxidizing nitrite.

Joshi (22) isolated a new organism that oxidized ammonia to nitrite. It did not grow in bouillon or gelatine and showed branching.

Hopkins and Whiting (20) isolated pure cultures of nitrite bacteria on silica jelly. The colonies were 1/8 inch in diameter, "colorless to opalescent at first and later orange yellow to brown." "When stained with gentian violet, they appeared as typical Nitrosomonas. Visible growth in solution was slow for the first forty days, but after that time a very profuse surface growth developed showing large blue colonies some of which were drawn up the sides of the flask by the surface tension of the liquid and there developed to a large size, (1/4 inch in diameter.)" This work has not been confirmed by any others.

Russel and Bartow (34) report the isolation of pure cultures of nitrifiers from activated sludge. They used washed agar and silicic acid gel as solid media. The silicic acid gel was prepared after the method of Temple and Stevens. They do not give details of their method of isolation or their tests for the purity of their cultures.

Bonazzi (7) isolated ammonia oxidizing organisms by using silicic acid gel. He described his organism as a Megalococci, 1.2 μ in diameter and of a slightly irregular roundish form. He noted that "when the cultures are in full and strong nitrification, the Megalococci give rise to the

small cocci." Some of these small cocci have been observed by him "to take up a gelatinous coating and revert to Megalococci." Neither of these forms could grow in bouillon.

Gibbs (16) gives a detailed review of the literature on nitrification. In his work he isolated Nitrosomonas and Nitrobacter. He used mainly silicic acid gel and washed agar as solid media. He observed sporadic growth in bouillon.

Fred and Davenport (14) reported the isolation of pure cultures of a nitrate forming organism by inoculating into washed nitrite agar various dilutions of so-called "enrichment cultures" which were in reality a combination of both enrichment cultures and sub-cultures. (At this point the writer wishes to differentiate between an enrichment culture and a subculture. An enrichment culture is obtained by constantly adding to a liquid culture obtained, fresh doses of medium as each previous dose is oxidized. A subculture is obtained by transferring to a fresh liquid medium a small inoculum from a culture which has recently completed oxidation. The above differentiation will apply throughout the following report.) The organisms obtained from the enrichment cultures did not show any visible growth in peptone beef infusion or "Heyden-Währstoff" solution. In form the organism "varied from a decided oval to almost spherical, while in size equally marked differences were seen." Mounts of zooglea-like masses with only a few loose cells in the field were obtained. Cells with flagella-like attachments were also observed.

Experimental

The purpose of this investigation was to isolate, if possible, in pure cultures the organisms that cause, first, the oxidation of ammonia into nitrite and, second, the oxidation of nitrite into nitrate. The methods adopted are those that have been used before. These methods have been modified from time to time as seemed necessary to facilitate the investigation.

Isolation of Nitrite Formers.

Crude Cultures:

The following nutrient solution #1 a modification of Gibbs (16) was used to isolate the organism causing the oxidation of ammonia.

Solution #1.

	gms.
$(\text{NH}_4)_2 \text{SO}_4$	1.0
K_2HPO_4	1.0
NaCl	2.0
MgSO_4	0.5
$\text{Fe}_2(\text{SO}_4)_3$	Trace
Water (conductivity)...	1000 c.c.

Solution #2.

MgCO_3	5.0 gms.
Water (conductivity)...	100 c.c.

In 10 E. flasks of 60 c.c. capacity, 5 c.c. of solution #1 and 0.5 c.c. of #2 were taken and a small chunk of greenhouse soil was added. The flasks were incubated at room

temperature and tested daily for nitrites. Dedidedly positive reactions for nitrite were observed between 5 and 6 days. Subcultures were then made by inoculating a loopful into flasks containing fresh solutions. These took about 15 days to show vigorous oxidation, when further subcultures were made by inoculating into fresh solutions. These subcultures were thus carried thru six generations. Nitrites were tested by Trommsdorf's reagents.

The vigorous oxidation was observed in about two weeks complete oxidation of $(\text{NH}_4)_2\text{SO}_4$ never took place. When cultures 8 weeks old were tested the presence of ammonia could still be detected by the use of Nessler's reagent.

In testing these crude cultures for purity Winogradsky's criterion that a pure culture of nitrifying organisms does not give growth in alkaline bouillon was used.

0.5 c.c. of liquid culture from the sixth generation of culture obtained above was inoculated into bouillon. In 3 days growth was noticed showing the cultures were not pure.

The liquid cultures were therefore carried thru further subcultures as described under oxidation experiments reported in part II. The last of these subcultures, generation 10, was tested in bouillon and found impure. It was decided then that bouillon sterile culture could not be obtained by the subculture method.

Experiments with Washed Agar.

The use of solid media as a means of obtaining pure cultures was then resorted to. The literature on the use of

solid media is quite extensive, but there is no method yet devised that gives a rapid growth and formation of large colonies of the nitrite formers.

The following experiments were planned to shorten the period of growth of the organism on solid media.

Washed agar was used as substratum. It was obtained by soaking agar in distilled water for 8 to 10 days. The water was changed every day. The agar was then dried at room temperature.

The most probable reason why an organism capable of such vigorous physiological activity in soil should make such slow growth in artificial media is the want of proper nutrients or the presence of toxic or mildly toxic substances in the medium. To remove, or make less effective, some of these inhibitory factors, the following combination of nutrient salts were tried:

Solution #3	gms. per 100 c.c.
(a) K_2HPO_4	1.5
(b) $(NH_4)_2SO_4$	1.5
$MgSO_4$	0.75
$Fe_2(SO_4)_3$	0.02
(c) $NaCl$	3.0
Na_2CO_3	1/5

This medium (16) was used by adding one c.c. of each of the above 3 solutions (a), (b) and (c) to 10 c.c. of washed agar. It gave a P_H value of 8.6. The reaction was determined by the "color standards" of Medalis (25,26).

Solution #4, same as #3 except that Na_2CO_3 was replaced by an excess of MgCO_3 . In practice sterile MgCO_3 powder was put into the petri dish and the other nutrients were later added. With 10 c.c. washed agar, this medium gives a P_H value of 8.8.

Solution #5, same as #3 except that Na_2CO_3 was replaced by an excess of CaCO_3 . With washed agar the reaction was P_H 8.2.

Solution #6, same as #3 except that both Na_2CO_3 and NaCl were replaced by CaCO_3 in excess.

Solution #7, same as #3 except that Na_2CO_3 was replaced by a mixture of equal quantities of MgCO_3 and CaCO_3 . The reaction with washed agar was P_H 8.0.

Solution #8a, same as #3 except that (a), (b) and (c) were mixed and sterilized together. The reaction with washed agar was P_H 7.6.

Solution #8b, same as #3 except that Na_2CO_3 was replaced by MgCO_3 and all three solutions were sterilized together. The reaction with washed agar was P_H 7.6

Solution #8c, same as #3 except that Na_2CO_3 was replaced by CaCO_3 , and all were sterilized together. The reaction was P_H 7.6.

Solution #9. Solution #3 concentrated 10 times.

Solution #10. Solution #3 concentrated 10 times and all three solutions sterilized together.

A subculture showing vigorous oxidation of ammonia was used as inoculum in the following experiments:

In each petri dish 0.5 gm. $MgCO_3$ was placed and a c.c. of each of the three solutions of #4 added. A loopful of the liquid culture was added to melted and cooled washed agar and poured into the plate. The plate was gently rotated so that the $MgCO_3$ was evenly distributed. The plates were incubated at room temperature.

After three weeks small speck-like colonies were observed. When tested for NO_2 by throwing a chunk of medium into Trommsdorf's reagent, a positive test was obtained. The colonies were carefully picked out under a dissecting microscope and each was inoculated into slightly alkaline bouillon. Growth was noticed within a week for all types of colonies.

The above experiment was repeated by using nutrient solutions #5, 6, 7, 8a, 8b and 10. The growth in plates was similar to the above and when tested for purity also gave growth in bouillon.

There were two types of colonies in these plates. Type 1 was deep, circular, small and pale blue with well defined edges, while Type 2 was surface spreading and almost colorless.

These colonies were then inoculated into washed agar plates containing solution #8b and also into flasks of nutrient solutions #1 and #2. Sterile air was bubbled thru the flasks. The object of bubbling the air thru being to increase the efficiency of the organism as was found in experiments described in part II.

The plates showed growth in 8 days and when the colonies were tested for purity they all grew in bouillon. The nutrient solutions inoculated with colony Type 1 gave oxidation in 12 days. From these, further subcultures were made by inoculating with a 25 c.c. of solution and bubbling air thru. Vigorous oxidation was noticed. Inoculation into bouillon from these liquid cultures gave growth. This growth was taken as an indication of in pure cultures.

Silicic acid gel.

Having failed to isolate the organism that causes ammonia oxidation by washed agar, silicic acid gel was next tried. The method adopted for its preparation is virtually the same as that employed by Gibbs (16). In dialyzing the silicic acid, it is essential that the atmosphere in the dialyzer be entirely devoid of CO_2 . By making use of a bell jar supplied with a membrane at the bottom and an opening at the top, it can be easily arranged to free the air of CO_2 . A 2.5 percent silicic acid solidifies with solutions #9 and 10 very readily. More dilute solutions sometimes failed to solidify and never solidified in a few minutes.

Beijerinck's (4) method of making silica plates by pouring into petri dishes equal amounts of equivalent solutions of HCl and Na_2SiO_3 was also used in part of this work.

A series of 7 experiments were made in which the inoculum used was obtained from liquid cultures that were obtained from plate cultures. In these plates growth was observed

in 4 to 20 days. Three types of colonies were observed. These differed from colony Types 1 and 2 previously described and consequently will be spoken of as Types 3, 4 and 5.

Colony Type 5 made its appearance first. It was irregular with a rough but somewhat flat surface. It had an erose edge and an amorphous internal structure. It was slightly yellowish in color. The organism was a coccus about $0.4 - 0.5\mu$ in diameter. The size of the colony was about 0.5 m.m. in diameter.

Colony Type 4 was a small circular colony not exceeding 50μ in diameter, greenish-yellow in color, with an amorphous internal structure. The organism was a coccus form, almost always enclosed with other coccus forms in sheaths.

Colony Type 3 was yellow in color and did not reach over a maximum of 200μ in diameter. It had a regular outline approaching a circle. It had a rough elevated surface and a finely granular internal structure. The organism was a coccus, varying in size from 1.5 to 1.0μ in diameter.

All three types of these colonies gave growth in bouillon. When tested for oxidizing power by throwing a small chunk of medium including the colony into Trommsdorff's reagents a positive test for NO_2^- was obtained.

In another series consisting of 13 experiments, these colonies were then inoculated into silica plates and nutrient agar plates. The method adopted for inoculation was to pick the colony with a chunk of medium and macerate it in a sterile covered

petri dish containing a few drops of solution #1, and then making streaks on the silica plates prepared according to Beijerinck.

In plates inoculated with Type 3 the test for NO_2^- was positive while in plates from colony Types 4 and 5 it was negative. Nutrient agar plates did not give any growth with Type 3 colony.

The colonies from plates inoculated with Type 3 were then again streaked on silicic acid plates and incubated at room temperature, but this time no oxidation of ammonia could be detected.

The colonies of all three types were inoculated ^{each} into liquid medium. Tests were made for NO_2^- every week for 8 weeks in all cases. Colonies of Types 5 and 4 did not show oxidation. Type 3 colonies gave oxidation only in 10 percent of the cases tried. In the cases of positive oxidation tests for purity gave growth in bouillon. The colony type used as inoculum for these liquid cultures did not give growth in media containing organic matter. These results from plates and liquid culture tests were not obtained from a single set, but from a series of experiments. This would indicate that the nitrite formers are either inconsistent in their action in bouillon or have undergone a metamorphosis in the liquid culture, thus showing quite conclusively that the power of these organisms to oxidize ammonium sulfate is sometimes lost when the organism is grown on a nutrient medium and in pure culture. It is also possible that there might be two different organisms in the same colony.

In the silicic acid $\text{Mg}(\text{NH}_4)\text{PO}_4$ plates the inoculum was made by the streak method and incubated at room temperature for a period of 4 weeks. The first difficulty encountered with this method was that it was not possible to see the colonies with the microscope on account of $\text{Mg}(\text{NH}_4)\text{PO}_4$ particles. The colonies were much slower to grow than in ordinary silicic acid plates. The oft repeated assertion that $\text{Mg}(\text{NH}_4)\text{PO}_4$ salts are dissolved and a light "halo" forms around colonies could not be verified. Typical colonies were marked and observations made for weeks without noting the $\text{Mg}(\text{NH}_4)\text{PO}_4$ crystals surrounding the colonies or crystals surrounded by colonies diminishing in size to any extent. When tested for oxidation by Trommsdorf's reagents the washed agar medium showed diffused blue for the presence of NO_2 . The silicic acid $\text{Mg}(\text{NH}_4)\text{PO}_4$ rarely gave positive results for NO_2 . The colonies for both the media gave growth in bouillon.

Use of Soil-Extract Washed Agar

Preparation of soil extract (40).

To one kilogram of soil two liters of water was added and steamed in the autoclave for two hours. The liquid was then pressed thru a Chamberlain filter and sterilized in the autoclave three times at intervals of 24 hours under pressure of 15 lbs. for 4 hours each time. The reason for sterilizing three times was to destroy the mold spores that were noticed to grow in solutions sterilized only once.

The washed agar was added at the rate of 20 grams per liter and tubes containing 10 c.c. prepared. The nutrient solution used in all the experiments with this medium was solution #3.

A vigorously oxidizing liquid culture generation 15, was used as inoculum. 1 c.c. of this culture was diluted 25; 625; 15625 times. Plates were made using a drop from the above dilution and incubated at room temperature. In 8 to 9 days the plates showed colonies, some of which could be seen with the naked eye while others could not. There were in all four different kinds of colonies. These colonies were separately picked by a micropipette (48) and added to 10 c.c. of soil extract washed agar. This was diluted by transferring a drop to another tube. Both were plated as above.

The colonies from these plates seen under the microscope in 10 days were tested for oxidation by adding a chunk of medium containing the colony to Trommsdorff's reagents. Some of the colonies showed the production of NO_2 by the formation of a blue color immediately around them. The organisms in these colonies were of two types, one a coccus form 1.0 to 1.2 μ , and another a small coccus 0.4 to 0.5 μ . The latter appeared to form chains with a sheath resembling the formation of conidia in actinomyces.

The liquid medium inoculated similarly and at the same time as these plates failed to show oxidation for nine weeks. Bouillon tubes made from colonies of the type that showed NO_2 production gave ^{50 percent} growth that is 50 percent purity. All the

colonies inoculated into the bouillon tubes looked alike.

The colonies which were similar to the ones that showed oxidation to Trommsdorff's test were picked out by the micropipette and again plated into soil extract washed agar. The resulting colonies were similar to the colonies used as inoculum and gave the oxidation test, but when inoculated into bouillon growth was observed. Thus indicating either the presence of contaminating organisms or that the bouillon test is not reliable.

This plating out method by picking out one single colony by a micropipette was carried on thru nine generations. The 9th generation showed colonies which were in all respects similar to the ones obtained in the first generation, gave a test for oxidation and were not absolutely pure according to the bouillon test. In one experiment 50% of the colonies inoculated gave bouillon sterile tests. In two of the experiments 25% gave tests of bouillon sterility. In others, bouillon sterility varying from 10 to 25% was noticed. Among the colonies that showed a test for NO_2 production, some were bouillon sterile while others were not. Plates that were bouillon sterile in one generation may or may not show growth in bouillon in the second generation. In short, the bouillon sterility test gave variable results, Under conditions made identical as far as possible and with colonies that were identical in morphology and ammonia oxidizing powers. The ability of these colonies to oxidize liquid medium was poor, a characteristic that has been observed by Winogradsky and Gibbs who ascribed it to the causes that one

colony is too small an inoculum and that the organism loses part of its vitality due to the conditions on the solid medium.

Dilution Method

On the assumption that in the colonies obtained in the above experiments two or three organisms were growing together the method of dilution was adopted to separate them. From the above plates a single colony was picked by a micropipette and forced out into 10 c.c. sterile water containing 2 grams sand freed of organic matter and all soluble material. The flask was vigorously shaken for 2 to 3 minutes and 1 c.c. removed and added to another sterile flask containing 9 c.c. water and sand. Thus dilutions up to 1/10,000,000 were made. A drop from these dilutions was used as inoculum and plates were made with soil extract washed agar. In some cases 1 c.c. of liquid from the 100,000, 1,000,000 and 10,000,000 dilutions were used as inoculum for the reason that when only a drop was used many plates failed to show growth.

and #2

Bouillon tubes and solution #1/were inoculated at the same time and with the same amount of inoculum as the plates. The plates were incubated from 4 to 8 weeks. In all of the 6 series of experiments the dilution of 10,000,000 never gave growth of nitrifiers or showed the presence of NO_2 as indicated by Trommsdorf's reagents. But this dilution always gave a growth of actinomyces.

Plates of 1,000,000 dilution often showed colonies that gave a positive test for NO_2 , but they always contained

actinomyces. The latter usually appeared in large numbers and grew larger after the plates had lost some of their moisture by evaporation. The total number of actinomyces colonies appearing in the above dilution was below 10. Dilutions of 10,000 and 100,000 always gave growth and oxidation and contained a smaller proportion of actinomyces on the plates. Except in a few cases, dilutions below 1,000 were too thickly sown and did not permit of picking individual colonies even with a micropipette.

Up to and including the 1,000,000 dilution the bouillon tubes gave growth.

Description of Colonies.

These colonies were very small and could be seen distinctly only by magnification. In the plates of lower dilution the colonies did not attain more than 50 μ in size. In dilutions of 100,000 and 1,000,000 colonies as large as 250 were observed. They averaged 150 μ in size.

No growth was observed in the plate during the first week. Later when the plate was held to the light the colonies appeared as tiny specks of cream color. Under a magnification of 135 they appeared yellowish. They were circular convex, with an erose edge that is very indefinite. The deep colonies were spindle shaped, but on coming to the surface they spread into a circular shape. The internal structure was granular and refractive.

A possible contaminating form surrounding these colonies as a thin spreader or film-like growth was often observed. It was always on the surface. It had a lacerate edge and amorphous internal structure. It was colorless. Thors larger than the central yellow colony it was also microscopic with a diameter rarely approaching 500 μ . It was not observed in early dilutions (1-1000) and was not found with deep colonies. It appeared when the deep colonies broke through the surface.

Liquid Cultures.

and #2
Solution #1 / inoculated with colonies from soil extract washed agar gave on the whole very satisfactory results. In 90% of the experiments conductivity water was used while soil extract was used in the rest. In 80% of the inoculations made one colony was carefully picked by a micropipette and then forced into the flask containing the liquid medium. In other cases 1 c.c. from dilutions of colonies described under "Dilution Method" were used as inoculum. In all other cases a chunk of medium including the colony was picked by a fine platinum needle and washed into the liquid.

All the flasks were incubated at room temperature from 4 to 12 weeks. Oxidation was noticed in some cases as early as two weeks, while in the majority of cases it took four weeks. This slow oxidation was especially noticed in cases where the single colony or part of the colony was picked out by the micropipette and inoculated into the liquid. In about 30% of the cases

oxidation failed entirely.

In cases where 1 c.c. portions from dilutions were used as inoculum oxidation took place up to 1,000,000 dilutions in cases where the original culture used for dilutions came from a liquid culture. In cases where a colony was macerated and diluted the oxidation was obtained up to 100,000 dilutions. The average time taken was four weeks. These liquid cultures when tested for purity as a rule gave growth in bouillon.

While the above tests show conclusively that the ammonia oxidation takes place in mixed cultures and further that the oxidizing organism cannot be isolated in pure culture, the writer is able to state definitely that the organism that causes ammonia oxidation is a coccus about 1.2μ in diameter. The size varies. It is not always regular in form being oval sometimes. No capsule was observed. In hanging drop the organism was not found to be motile. It stains well with cold saturated aqueous fuchsin in 15 seconds.

Effect of CO₂ on Growth of Organisms

In order to see if the conditions favorable to the growth of nitrite formers and unfavorable to the growth of contaminants could be created, the following experiments were conducted.

Two liquid cultures and one plate culture colony were diluted as described previously up to 10,000,000 and used as

inoculum for making soil extract washed agar plates. Sterile sand water was used to make the dilutions. Solutions #1 and 2 were inoculated in 10 c.c. portions with the various dilutions.

One series of dilution plates were incubated in desiccators where normal air conditions prevailed. Another series was incubated in desiccators in which was placed NaOH to absorb the CO₂. The top of the desiccator was sealed tight with vaseline. A third series was incubated in desiccators from which air was driven out by the action of H₂SO₄ on Na₂CO₃ thus creating an atmosphere containing a large excess of CO₂. In a fourth desiccator pyrogallie acid and Na₂CO₃ were used and oxygen was removed. The plates and the flasks were examined at the end of every four weeks.

The results tabulated below reveal very clearly the effect of CO₂ on the oxidation of (NH₄)₂SO₄ by the organism. In plates incubated without CO₂ no oxidation was observed when tested with Trommsdorff's reagents.

Effects of CO₂ on the Action of Ammonia Oxidizer

Series No.	Source of inoculum	Normal Air	Normal Air without CO ₂	Soil extract washed agar	Washed agar	Air displaced by CO ₂	Normal air without Oxygen	Liquid Cultures under Normal air conditions.
<u>Plate cultures</u>								
Dilutions up to and in which NO ₂ was present								
681	Liquid culture	1,000	-	-	-	10,000	-	1,660,000
682	Liquid culture	1,000	-	-	-	10,000	-	100,000
683	Washed agar plate	1,000	-	-	-	1,000	-	100,000

In plates where excess of CO_2 was present there was oxidation in greater dilutions than under normal air conditions. In plates deprived of oxygen the organisms failed to oxidize $(\text{NH}_4)_2\text{SO}_4$. It was also clear that excess of CO_2 promoted growth of nitrite formers and showed NO_2 in higher dilutions than did normal air. In series 681 both washed agar and soil extract washed agar plates were incubated in CO_2 - free air. Neither of the plates gave oxidation showing that soil extract could not act as a source of carbon for nitrite formers and sustain oxidation in cultures where free CO_2 was not available.

The nutrient solution #3 used for these plates contained a basic carbonate but that evidently does not serve as a source of carbon for ammonia oxidizers. It is free CO_2 that the organism uses. This is in conformity with the findings of Winogradsky and Omeliansky (47, Godlewski (17, 18, 19), and Bonazzi (8.)

Nitrate Formers

The following nutrient solution (16) was used to isolate the nitrate former in soil.

Solution #11:

NaNO_2	1.0	gm.
Na_2CO_3	1.0	"
K_2HPO_4	0.5	"
NaCl	0.5	"
MgSO_4	0.3	"
$\text{Fe}_2(\text{SO}_4)_3$	trace	
Water	1000	c.c.
(conductivity)		

Enrichment cultures:

The solution was sterilized for 15 minutes under 15 lbs. pressure and 25 c.c. portions placed in 250 c.c. sterile Erlangmeyer flasks. The flasks were inoculated with 1 gram of soil. Fifteen flasks were inoculated.

The presence of nitrate was tested by diphenylamine. In 10 days NO_2 was oxidized to NO_3 in almost all the flasks. As the NO_2 disappeared 1 c.c. of this culture was inoculated into 25 c.c. of fresh solution. To the mother culture 5 c.c. of fresh NO_2 solution was added and again incubated. When all NO_2 again disappeared, further addition of NO_2 was made. This was carried on 25 times. As each addition of solution was oxidized fresh solution was added. The average time taken to oxidize 5 c.c. of the solution was 4-5 days.

The cultures were then tested for contaminating forms by inoculating into slightly alkaline bouillon. Growth was given by all the 15 cultures.

Subcultures:

The subcultures obtained by inoculating fresh NO_2 solution with 1 c.c. of the first enrichment culture were carried thru 17 generations. The time required to oxidize 25 c.c. of solution was much greater in this case than in the enrichment cultures. The average time taken for each generation varied from 9 to 16 days. Fifteen cultures were carried.

- Tests were made of the 17th generation for purity by inoculating into alkaline bouillon. All the 15 cultures gave growth.

Dilution Experiments

Fifteen cultures of nitrite oxidizers which were carried thru 8 subcultures were diluted 25, 625, 15,625, 390,625 and 9,765,625 times. One c.c. from these dilutions was inoculated into tubes of "Nährstoff-Heyden" solution, bouillon solution, and 10 c.c. of solution #11. The cultures were incubated at room temperature.

The first three solutions gave growth in "Nährstoff-Heyden" solution and bouillon. The last two dilutions failed to grow in "Nährstoff-Heyden," but gave growth in most cases in bouillon. The dilutions that did not give growth either in bouillon or in "Nährstoff-Heyden" often failed to oxidize NO_2 . The dilutions that failed to grow in bouillon and "Nährstoff-Heyden," but oxidized NO_2 in solution were inoculated into fresh

solution #11, bouillon and "Nährstoff-Heyden" solution.

NO_2 was oxidized, but growth was noticed in bouillon and "Nährstoff-Heyden" solution. This was another instance where the mother culture failed to grow in bouillon while the daughter culture did. Yet both oxidized nitrite. This seemed to indicate that the bouillon test for the purity of these cultures is not constant and conclusive.

Experiments with Washed Agar and Silicic Acid

Gel Solid Media.

Attempts were made to grow the liquid culture organisms on solid medium. Washed agar was used along with silicic acid gel. A loopful of inoculum was added to each 10 c.c. of agar and dilutions made as usual. In silicic acid a loopful of inoculum was added to each plate. Plates were incubated at room temperature. In three weeks light yellow microscopic colonies about 500 μ in size appeared. The surface colonies had somewhat indefinite edges with a heart shaped granular center. These colonies were picked off with a fine platinum needle and inoculated into 5 c.c. of solution #11. Presence of NO_3 was shown in some of the flasks in 7 days. Some of the organisms had oxidized all the nitrite in 16 days. From these liquid cultures subcultures were made. A loopful of inoculum was added to a 60 c.c. Erlenmeyer flask containing 5 c.c. of solution #11. The flask was well shaken and a loopful from this was added to another flask. Five such pairs were made and incubated. Complete oxidation took place in two weeks in the flasks receiving a loopful of inoculum direct

from the mother culture. In dilution cultures 19 to 21 days were required for oxidation. Subcultures were further made from these by loopful inoculations and carried thru 6 to 8 generations.

The solutions were then tested for purity. They all gave growth in bouillon. Plates were made from F₃ generation and the colonies were inoculated into bouillon. Growth was given in 3 days.

The organisms that grew in bouillon when inoculated into solution #11 failed to oxidize.

Stains made from liquid cultures showing vigorous oxidation of nitrite to nitrate showed short rods measuring about 0.8 μ long and 0.4 μ broad. Cold saturated aqueous fuchsin was used. In hanging drop no motility was observed. Stains for flagella were not secured.

Action of Nitrifying Organisms in Sand as Substratum

The liquid cultures create conditions very different from those that exist in soil. To study in the laboratory the activities of nitrifying organisms under conditions as nearly as possible similar to those that exist in soil many devices have been suggested. Bazarewski (1) used sand cultures to test the effect of organic matter on the nitrifying organisms. Coleman (11) worked with the nitrifiers with sand and soil as media. Wimmer (43) used sand to test for the toxic effect of

organic matter on nitrifying organisms and secured good results.

Increase in the nitrifying power of a soil by inoculating it with pure cultures of nitrifying organisms is a possibility that is suggested by the practice of inculcating soil for legume bacteria. The following experiments were carried out to obtain indications as to the possibilities along this line.

Sand Inoculation

As a preliminary test sand washed clean with dilute HCl and distilled water was sterilized in petri dishes. One c.c. of liquid culture of nitrite former and 1 c.c. of nitrate former were added to 100 c.c. of solutions # 1 and 2 and shaken well. Enough of this was added to each petri dish to bring the moisture content of sand to the optimum and incubated at room temperature.

Tests were made for NO_2 and NO_3 at frequent intervals. In 10 days traces of NO_2 were noticed in sand. In 14 days both NO_2 and NO_3 were observed. In 18 days NO_2 had disappeared in 75% of the plates and only NO_3 was present.

To find if similar action takes place in deeper layers of sand, the experiment was repeated in beakers 6 inches deep and in 250 c.c. Erlenmeyer flasks. Results almost similar to those obtained in petri dishes were obtained here also.

Action of Nitrifiers in Presence of Growing Plants.

The favorable results in rapid oxidation of ammonia nitrogen to nitric nitrogen led to the following experiment.

The apparatus here employed is on the same lines as that of Fred (13).

A battery jar (Fig. 1) was fired with a specially made cover PP of burnt clay. The cover was provided with a groove AA into which the battery jar edge JJ could stick. In the center of the cover there was a hole B 1.5 cm. in diameter and around this hole a groove CC 6.5 cm. deep and 5 cm. in diameter. There were two other holes D and E 2 cm. in diameter placed near the edge of the cover. The diameter of the jar was 14.5 cm. and the height 18 cm. In the groove A between cover and the battery jar cotton was placed to avoid bacterial passage. Thru D passed a tube bent at right angles and reaching the bottom of the jar. Thru E passed a similar tube but extending only 3 cm. into the jar. Around tubes D and E cotton was placed to keep off outside organisms. The outer ends of tubes D and E were provided with rubber tubing M and N and screw clamps S and T. Thru the central hole B 500 grams of clean, washed dry gravel was poured into the jar and evenly distributed on the bottom. On top of it 2750 grams of sand cleaned in dilute HCl and washed in distilled water and dried was poured in. The surface of sand came very near the top of the jar. A glass cylinder GG, 5 cm. in diameter and 20 cm. in length, fitted into groove CC. A glass tube H 1.5 cm. in diameter and 25 cm. in length passed thru B and rested on the sand. The top of this tube carried a cotton plug. Between the two cylinders there were loose cotton packings W_1 and W_2 at top and bottom and three

glass rods KK about 0.4 cm. in diameter and 20 cm. in length.

The entire apparatus was sterilized in the autoclave 3 times on alternate days at 15 lbs. pressure for 3 hours. The sterility of sand was tested by taking a small amount of sand with a sterile needle and inoculating into "Nährstoff-Heyden" agar.

Solution used:

The solutions that produced the maximum growth of wheat according to Skinner and Reid (38) contained phosphate, nitrate and potash in the ratio of 20 - 40 - 40. In this work $\text{CaH}_4(\text{PO}_4)_2$, NaNO_3 and K_2SO_4 solutions with a concentration of 160 parts per million of P_2O_5 , NH_3 and K_2O were made. The solutions were sterilized separately and then mixed in equal parts. For every 100 c.c. of the solution, 1 c.c. of the liquid culture of nitrite former and 1 c.c. of nitrate former were added and well mixed. Enough solution was added thru tube D to the battery jars to bring the moisture content of the sand to the optimum.

Wheat seeds were sterilized by washing in 1 - 500 HgCl_2 solution, 50% alcohol and sterile water. The cotton plug was removed from tube H and two seeds were dropped in. When the seedlings showed up above the cotton packing W_2 , the tube H was slowly pulled up and the cotton was packed around the seedling by the glass rods KK. The jars were then removed to the greenhouse.

Sterile water was added thru D as evaporation took place. The seedlings grew to maturity and formed heads as under

normal conditions.

At the end of the growing period the sand was tested for nitrates, nitrites and ammonia. Large amount of nitrates and traces of ammonia were present. The growth of the plants and the presence of nitrates indicate the ability of the nitrifying organisms to function in the presence of growing plants.

Contaminating forms

Bacterial forms:

It has been the experience of all who have tried the isolation of nitrifying organisms that contaminating forms persist both in cultures of nitrite and nitrate formers. Berstyn (5) found in his *Nitrobacter* cultures of F₂₀ generation, Bac. comes, Bac. Modestum, Bac. debile and Pseudomonas humicola. Beijerinck (4) found in his Nitrobacter cultures, Bacillus Nitroxus. This organism proved very difficult to be separated from Nitrobacter.

Gibbs (16) found in his cultures of Nitrosomonas and Nitrobacter, three organisms. One he described as a yellow pigment forming very small bacillus, about 0.4 μ wide and 1.0 μ long; always single never found in chains. The second was a very small coccus, or sometimes slightly oval, about 1 μ in length often found in pairs. Both organisms were gram negative. The

third form was a minute bacillus, producing a slight turbidity in bouillon after incubation of several days.

The organisms that contaminated the nitrate formers, as mentioned above, are more or less fully described, but the literature does not contain a detailed description of the organisms that contaminate nitrite formers. In this work the organisms contaminating the nitrite former are described.

In the earlier part of this work it has been shown that the nitrite former lived in close association in the same colony with other non-ammonia oxidizing organisms. The latter made good growth in bouillon. Two kinds of growth were noticed in bouillon when it was inoculated with colonies of nitrite formers. There was a slight turbidity in liquid accompanied by the formation of slimy film at or near the surface. The other growth was much heavier consisting of coarse orange granules and a very thin bluish film climbed the sides of the tube. The former is a coccus form and the latter is a short rod.

Coccus:

Morphology:

Form: The organism was definitely spherical in shape.

Size: 48 hour agar cultures of the organism showed individual cells the most of which were from 0.4 to 0.5 μ in diameter. Often these were found in pairs. The size of the organism varied little with age or change in media. On potato the organisms appeared in chains of 5 and 4, often bent and covered with a sheath-like membrane.

Motility: Hanging drop cultures were examined from bouillon cultures. Motility was not observed. Flagella stains were

not obtained.

Staining reactions: The organism was easily stained by ordinary stains. Saturated aqueous fuchsin gave good stains in 15 seconds. Stains made from potato cultures showed chain-like formation enclosed in sheaths. The sheath was easily decolorized by absolute alcohol. The gram stain was positive.

Spore formation: None of the stained preparations suggested spore formation, and both young and old cultures failed to resist 80° C. for 10 minutes.

Cultural characteristics:

Agar streak: There was scanty microscopic growth on agar streak cultures. Very small colonies grew along the streak and did not run together. The colonies were convex, refractive, light yellow or colorless. No odor was observed. The growth was brittle.

Agar plate colonies: In 10 days the colonies reached the size of about 300 . They were oval and heart shaped with rough and raised surface. The edge of the colonies was entire and their internal structure granular.

Nutrient broth: The reaction of the broth used was slightly alkaline. In 2 or 3 days a slight turbidity in the liquid with a thin film on the surface was noticed. Later the film grew heavier and on shaking fell to the bottom of the tube. It was mucilagenous and appeared floccy. It stuck to the platinum needle and was difficult to be torn off. It was yellowish in color.

Nutrient agar plate colonies: Growth slow, form circular or oval,

surface rough, elevation raised, edge erose, internal structure coarsely granular, size in 12 days 140 μ .

Gelatin stab: Thin bluish growth on surface next to walls of tube was seen in 12 days; no growth along the line of puncture. In 4 to 6 weeks old cultures the growth was heavier and yellowish granular. No liquefaction.

Gelatin plate colonies: In 3 days colonies 35 - 100 μ big were observed. They were circular and raised with an entire edge; no liquefaction; internal structure first amorphous later finely granular. The colonies were refractive and light yellow in color.

Dextrose agar slant: Growth moderate; form echinulate; elevation flat; luster glistening; topography contoured; chromogenesis grayish or cream colored; odor absent; consistency viscid; medium not changed.

Physiology.

<u>Chromogenesis:</u>	Nutrient broth	: light yellow
	Nutrient gelatin	: bluish first and : then light yellow
	Nutrient agar	: none
	Potato	::light yellow.

Production of indol in bouillon was observed in 6 days.

Relation to oxygen: The organism was an obligate aerobe.

Fermentation: The organism grew well in lactose, dextrose, saccharose and glucose; slight acid was observed in last two media. Gas was not produced. The growth was similar to that in bouillon.

Milk: In two weeks milk was curdled and peptonized.

Nitrate reduction: Was not noticed tho small amount of fleecy growth was observed at bottom of tube.

Diastatic action was not observed.

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Short rod B.

Morphology:

Form: The organism is a short rod.

Size: 48 - 72 hour cultures of the organisms showed individual cells, the most of which were from 0.6 to 0.7 μ in length, and 0.5 to 0.6 μ in width.

Arrangement: Cultures of all the organisms showed many isolated cells, altho pairs were very frequent and chains of 3 or 4 were also common.

Motility: Was not observed in hanging drop cultures or by dark ground illumination method and stains for flagella were not obtained.

Staining reactions: The organism stained readily with the ordinary stains. Saturated aqueous fuchsin was mostly used in this work.

Spore formation: Was not observed in any of the stained preparations and both young and old cultures failed to resist 80° C. for 10 minutes.

*According to the Descriptive Chart adopted by the Society of American Bacteriologists, Dec. 30, 1920.

Cultural characteristics.

Agar stroke: Growth scanty and microscopic; filiform and beaded; elevation flat; luster dull; topography slightly bullate; refractive; consistency brittle; chromogenesis dull cream color; no coloration of medium; rate of growth slow - only slight growth being observed in 3 days.

Agar plate colonies: Growth slow and punctiform; surface rough; elevation convex; edge erose; internal structure coarsely granular.

Gelatine stab: Orange granular growth on surface and none along the line of puncture; liquefaction not observed; medium not colored; rate of growth slow - growth not being noticed in three days; in two weeks the amount of growth increased and began to climb the walls of the tube.

Gelatine plate colonies: Observed in 3 days; form irregular; size 200 - 300 μ ; some aggregates of colonies reached in size 1 to 2 mm.; elevation convex; edge erose; liquefaction none; internal structure coarsely granular.

Nutrient broth: The medium used was slightly alkaline in reaction. On the surface orange-yellow granules formed and a thin pale film that climbed up the sides of the tube. Clouding in the liquid was not noticed unless the tube was shaken. There was no odor or sediment; the granules fell to the bottom but none grew at the bottom. Rate of growth was observed in 48 - 72 hours.

Dextrose agar slant: Growth abundant; form effuse; elevation raised; dull appearance; topography bullate; opaque; chromogenesis orange yellow; odor none; consistency brittle.

Physiology:

Chromogenesis: Orange yellow on nutrient broth, nutrient gelatine and potato.

Production of indol: Observed in six days.

Relation to oxygen: obligate aerobe.

Diastatic action: Slight.

Fermentation: Organism grows well in lactose, dextrose, saccharose and glucose, but without producing gas or acid. As in bouillon the growth is at the surface while the liquid remains clear.

Milk: On the surface orange granular growth was observed, but there was no action on the medium.

Nitrate reduction: Not observed.

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Actinomyces are consistently found among the contaminating forms in both the liquid and solid cultures of nitrifying bacteria.

Beijerinck (4) working with Nitrobacter came across A. robur Krainsky, A. grisens Krainsky and occasionally he noticed the presence of A. diastaticus Krainsky and A. cellulosee Krainsky. He claimed these organisms living in inorganic media used for nitrifying organisms obtained their food from the organic matter present in the laboratory air. Beijerinck found actinomyces usually in the film (Haut) formed on the surface of old cultures. A similar observation was made in the nitrate former cultures obtained in this work.

No other investigators have observed actinomyces in their cultures of nitrifying organisms. The Hyphomicrobium of Stutzer and Hartleb (40) in its morphological characteristics reminds one of the actinomyces. This organism of Stutzer and Hartleb did not give growth in bouillon at all, while the actinomyces gave growth at the top and also at the bottom of bouillon tubes. Neither of these organisms caused turbidity in alkaline bouillon.

In the experiments with nitrite formers the presence of actinomyces has been frequently noticed. In a plate of soil extract washed agar inoculated with a single colony of nitrite former picked out with a micropipette, colonies of actinomyces were noticed beside the colonies that resembled the mother colony. The latter were tested for NO_2 and positive results

obtained. They were subjected to further subculturing using a single colony picked out by a micropipette as inoculum. The last and 9th generation showed the same characteristics as the first.

The method of dilution of a single colony proved that the actinomyces was present in the colonies of nitrite formers in great numbers. In five generations of dilution cultures ranging up to 10,000,000 dilutions it was observed that in dilutions of 10,000 and above the number of actinomyces colonies increased while in the lower dilutions the colonies resembled the original colonies. The actinomyces when inoculated into soil extract washed agar plates did not show any oxidation. The typical ammonia oxidizer colonies when inoculated by making dilutions gave actinomyces in higher dilutions and true colonies in lower dilutions. This seemed to indicate that in the lower dilutions the growth of actinomyces was inhibited.

It was thought desirable to study these actinomyces in detail and therefore six of the most typical actinomyces colonies that were observed in all the cultures above studied were picked and their cultural; morphological characteristics determined. The method of study adopted here is virtually the same as that of Waksman (41).

Actinomyces B3

I. Morphology.

1. Spirochs: Short, straight, branching mycelium $0.6-0.7\mu$
x $5-7\mu$ Spirals not observed.
2. Conidia. Egg-albumen agar; oval 0.4μ x 0.6μ .

II. Cultural characteristics.

1. Synthetic agar.
Growth: Moderate spreading, colonies less than 1 mm.
Aerial mycelium: in clumps, white in color.
Soluble pigment - none
2. Calcium acetate - glycerine agar.
Growth: Scanty
Aerial mycelium: White.
Soluble pigment: None
3. Nutrient agar.
Growth: Growth moderate in media - colonies appear
like specks.
Aerial mycelium: Cream white specks.
Soluble pigment: None.
4. Loeffler's blood serum:
Growth: Scanty growth.
Aerial mycelium: White specks.
Soluble pigment: Brown.
Liquefaction: Rapid.

5. Starch agar.

Growth: Small specks on surface; no growth seen
below surface of medium.

Aerial mycelium: Short broken white clumps.

Soluble pigment: None

Diastatic action: Very good.

6. Potato.

Growth: None

7. Gelatine stab.

Growth: Numerous cream white colonies in a ring at
the surface, some at bottom.

Aerial mycelium: White.

Liquefaction: Rapid.

8. Litmus Milk.

Growth: Small colonies in the medium on the sides
of the test tube; small amount of sediment.

Aerial mycelium: None.

Peptonization: None.

9. Plain Milk.

Growth: Small colonies in liquid at surface and at
bottom.

Aerial mycelium: None.

Coagulation: None.

Peptonization: None.

10. Lactose broth:
Growth: Slight or none in liquid.
11. Dextrose broth:
Growth: Abundant at top; liquid clear.
Aerial mycelium: White.
Soluble pigment: Pink brown.
12. Dextrose slant:
Growth: Effuse surface growth. Wrinkled and leathery.
Aerial mycelium: Brown.
13. Dextrose agar plate:
Growth: Small speck-like growth.
Aerial mycelium: White.
Soluble pigment: Yellow.
14. Glycerine asparaginate agar:
Growth: Spreader in medium.
Aerial mycelium: White specks
Soluble pigment: None
15. Egg albumen agar:
Growth: Compact center colonies with fern-like edges.
Aerial mycelium: White specks.
Soluble pigment: None.
16. Glycerine nitrate agar:
Growth: Light yellow scanty growth.
Aerial mycelium: Cream white.
Soluble pigment: None

17. Starch gelatine stab(pH 6.4)

Growth: Speck-like colonies at top; heavy
sediment at bottom.

Aerial mycelium: None.

Soluble pigment: Light yellow.

Change of reaction: Alkaline.

18. Czapek's modified or synthetic solution:

Growth: Numerous speck-like colonies at top, in
the liquid, on the wall and at bottom.

Aerial mycelium: White.

Soluble pigment: Yellow.

19. Bouillon:

Growth: At bottom - numerous small speck-like
colonies - some on surface wall - liquid
clear.

Aerial mycelium: None.

Soluble pigment: Yellowish-orange.

20. Nitrate broth:

Growth: Small colonies all thru the liquid and on
the wall above the surface.

Aerial mycelium: Small white specks.

Soluble pigment: Yellow.

Nitrate production: Heavy.

Actinomyces #11

Morphology:

- I. 1. Spirals: Usually none on media studied. Mycelium consists of straight branching hyphae. Occasionally an open spiral is noted.
2. Conidia: Synthetic agar. Oval $1.3 \times 0.7\mu$. Also observed on dextrose agar, glycerine asparaginate, and glycerine nitrate agar.

II. Cultural characteristics:

1. Synthetic agar.

Growth: Compact colonies with few mycelia sticking out like bristles. Size of colonies less than 1 mm.

Aerial mycelium: White and straight.

Soluble pigment: None.

2. Calcium acetate glycerine agar:

Growth: Spreads below the surface speck-like colonies.

Aerial mycelium: White, long and scanty.

Soluble pigment: None, sometimes rose.

3. Nutrient agar:

Growth: Colonies 2 - 3 mm. in size. Abundant growth below surface.

Aerial mycelium: White.

Soluble pigment: Dark brown, sometimes none.

4. Loeffler's blood serum agar:

Growth: Effuse. Yellowish colored glossy surface.

Aerial mycelium: White or cream colored.

Soluble pigment: Slight yellow.

Liquefaction: Slow.

5. Starch agar plate:

Growth: Abundant spreader in medium.

Aerial mycelium: White.

Enzymatic action: Good.

6. Potato:

Growth: None at room temperature, but at 35° C.

echinulate growth in two weeks.

Aerial mycelium: White and gray.

Color of potato: No change.

7. Gelatine stab:

Growth: At top; after liquefaction sediment is
noted at bottom.

Aerial mycelium: White.

Soluble pigment: Yellowish brown.

Liquefaction: Rapid.

8. Litmus milk:

Growth: On surface - small amount of sediment.

Aerial mycelium: White.

Coagulation: Slow.

Peptonization: Slow; complete in two weeks.

Change of reaction: Alkaline.

Soluble pigment: Lavender.

9. Plain milk:

Growth: At surface - small amount of sediment.

Aerial mycelium: White.

Coagulation: Slow.

Peptonization: Complete in nine days.

Change of reaction: Alkaline.

Soluble pigment: Brown

10. Lactose broth:

Growth: Abundant on surface.

Aerial mycelium: White.

Soluble pigment: None, sometimes brown.

11. Dextrose broth:

Growth: Abundant on surface.

Aerial mycelium: White.

Soluble pigment: Dark brown.

12. Dextrose slant:

Growth: Echinulate.

Aerial mycelium: Cream white.

Soluble pigment: None.

13. Dextrose agar plate:

Growth: Abundant spreader in medium.

Aerial mycelium: White. (Bristling)

Soluble pigment: Yellow.

14. Glycerine asparagin agar:

Growth: Inside medium.

Aerial mycelium: White specks.

Soluble pigment: None.

15. Egg albumen agar:

Growth: In medium.

Aerial mycelium: White.

Soluble pigment: None.

16. Glycerine nitrate agar:

Growth: Heavy & colonies run together - spread
in medium.

Aerial mycelium: White.

Soluble pigment: Purple (iodine) sometimes none.

17. Starch gelatine stab: (pH 6.4)

Growth: At the bottom and on surface of liquid.

Aerial mycelium: None.

Soluble pigment: None.

Change in reaction: Alkaline.

18. Czapek's modified or synthetic solution:

Growth: Cream colored surface ring and small
amount of sediment at bottom.

Aerial mycelium: Cream white.

Soluble pigment: Light red.

19. Bouillon:

Growth: Small surface ring: (Liquid clear)

Aerial mycelium: White.

Soluble pigment: Reddish brown.

20. Nitrate broth:

Growth: Speck-like colonies on surface; some in medium.

Aerial mycelium: White or cream white.

Soluble pigment: Light yellow, sometimes rose.

NO₂ Production: None.

Actinomyces #12.

I. Morphology:

1. Spirals: None on the media studied; the aerial mycelium consists of only long straight branching hyphae.

2. Conidia: Oval 0.7 - 0.8 μ x 1.0 - 1.2 μ .

II. Cultural characteristics:

1. Synthetic agar:

Growth: Abundant spreading growth; penetrating into the medium.

Aerial mycelium: White and gray.

Soluble pigment: None.

2. Calcium acetate-glycerine agar:

Growth: Scant and slow.

Aerial mycelium: White.

Soluble pigment: None.

3. Nutrient agar:

Growth: Compact colony, slight penetration into medium, membranous on surface.

Aerial mycelium: White.

Soluble pigment: Whitish brown.

4. Lowffler's blood serum:

Growth: Glossy round yellowish colonies with depression in the center.

Aerial mycelium: None.

Liquefaction: None.

5. Starch agar:

Growth: Spreading in the medium.

Aerial mycelium: Cream white.

Soluble pigment: None.

6. Potato:

Growth: None at room temperature, but at 55° C. echinulate growth.

Aerial mycelium: White, heavy and all over growth.

Color of potato: Unchanged.

7. Gelatine:

Growth: Formation of heavy surface ring and a heavy sediment.

Aerial mycelium: None.

Soluble pigment: Orange yellow.

Liquefaction: Rapid.

8. Litmus Milk:

Growth: Surface ring of small colonies.

Aerial mycelium: None.

Coagulation: Slow.

Peptonization: Slow, first observed after 4 weeks.

Change in reaction: None.

9. Plain milk:

Growth: Slow and scant - not noticed for 4 weeks.

Aerial mycelium: None.

Coagulation: After 6 weeks.

Peptonization: None.

10. Lactose broth:

Growth: Heavy mat of colonies on surface, the liquid being clear and no sediment.

Aerial mycelium: White and all over the growth.

Soluble pigment: None.

11. Dextrose broth:

Growth: Heavy mat of colonies on surface, the liquid being clear; no sediment.

Aerial mycelium: White all over the growth.

Soluble pigment: None.

12. Dextrose slant:

Growth: Echinulate.

Aerial mycelium: White and gray - raised.

Soluble pigment: None.

13. Dextrose agar plate:

Growth: Heavy - spreading in the medium and on the surface.

Aerial mycelium: Chalk white.

Soluble pigment: None.

14. Glycerine asparaginate agar:

Growth: Radiating.

Aerial mycelium: White.

Soluble pigment: None.

15. Egg albumen agar:

Growth: Heavy; spreading in medium.

Aerial mycelium: White and gray.

Soluble pigment: None.

16. Glycerine nitrate agar:

Growth: Compact colonies.

Aerial mycelium: White, straight, projecting from colonies like bristles.

Soluble pigment: None.

17. Starch gelatine stab (pH 6.4):

Growth: None at surface, but 2 or 3 colonies about 1 cm. in diameter at bottom.

Aerial mycelium: None.

Liquefaction: None.

Change of reaction: None.

18. Czapek's modified or synthetic solution:

Growth: Heavy mat of small colonies on surface; colonies all over the surface of tube in liquid and a mat of colonies at bottom.

Aerial mycelium: White and gray.

Soluble pigment: None.

19. Bouillon:

Growth: One big colony covering the whole surface of liquid which is clear.

Aerial mycelium: White.

Soluble pigment: Orange brown.

20. Nitrate broth:

Growth: Colonies floating in the liquid and some sticking to the bottom of tube; liquid clear.

Aerial mycelium: None.

Soluble pigment: Light green.

NO₂ production: Heavy.

SUMMARY

In spite of the extensive work done in the isolation of nitrifying organisms, the methods are quite unsatisfactory. In this work the isolation of nitrite formers was attempted, first, by carrying on a long series of subcultures which failed to give absolutely bouillon sterile cultures. The contaminating forms were present with the nitrite former in the loop inoculum. The subculture method was modified by diluting the cultures from 1 to 10,000,000, and using the dilutions as inoculum. This method also proved inadequate. Next solid media namely, washed agar, soil extract washed agar, and silic^{ic}/acid gel were used. Different combinations of nutrient salts were tried with these media. Soil extract washed agar gave the most rapid growth of the organisms and oxidation of ammonia. But the colonies on this medium, like those on others, contained nitrite formers that gave sporadic growth in bouillon. This was proved by picking out a single colony by a micropipette, breaking the organisms loose in sand water blanks and diluting from 1 to 10,000,000 times and inoculating on to soil extract

washed agar plates. The growth in the plates contained besides colonies of the type used originally, actinomyces and two bacterial forms. All of these contaminators are described.

The fact that actinomyces always appear in the higher dilutions and are always closely associated with nitrite formation appears to be quite significant. No difficulty was encountered in securing pure cultures of these organisms, but they never showed any power of oxidation. They would grow in bouillon at the surface and sometimes at the bottom, but never produced any turbidity. There is a strong indication that a close relationship of some kind exists between these forms and the bacteria. More work will be required to show whether this relationship exists, if so, whether or not it is of a symbiotic nature.

The nitrite formers were grown in atmosphere with and without CO₂ and oxygen. Oxidation of ammonia was not noted either where CO₂ or O₂ was absent.

Isolation of the nitrate former was carried out on the same lines as those adopted in the isolation of nitrite former, making preliminary enrichment cultures, then sub-cultures and dilution cultures and finally inoculating these into two solid media, namely, silicic acid gel and washed agar.

Nitrite formers and nitrate formers in liquid cultures were inoculated into sterile sand supplied with nutrient salts. $(\text{NH}_4)_2\text{SO}_4$ Wheat seeds were planted. The ammonia was oxidized to nitrate and the wheat plant made normal growth.

CONCLUSIONS.

1. The bouillon test for purity of nitrite formers and nitrate formers is not conclusive due possibly to the fact that the organisms develop tolerance for bouillon.
2. The nitrite former may rapidly lose its power of oxidation of ammonia when grown in pure cultures.
3. Free CO_2 is essential for oxidation of ammonia by nitrite formers.
4. Air with large excess of CO_2 promotes ammonia oxidation better than normal air.
5. Nitrite formers grow most rapidly in soil extract washed agar. The carbonaceous material in soil extract does not act as a source of carbon to nitrite formers when free CO_2 is not available.
6. Pure cultures of nitrifying organisms oxidize ammonia to nitrite and nitrite to nitrate in the presence of growing wheat plants.
7. Use of $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ for the isolation of nitrite formers is not reliable.

PART II.

OXIDATION OF AMMONIA AND NITRITES BY MICRO- ORGANISMS UNDER DIFFERENT CONDITIONS.

Investigators have realized from the beginning that for vigorous nitrification both in the field and in the laboratory proper conditions of aeration and reaction of medium must be present. As early as 1877 (35) when the study of nitrification was still in its pioneer stage, Schloesing and Muntz observed that for strong nitrate production an abundant supply of air is necessary. They wrote two years later (36) that the access to oxygen is an essential condition for vigorous nitrification. Schloesing (37) determined the amount of oxygen necessary to oxidize " NH_3 to NO_5 " and found a definite ratio. Winogradsky (44) writing on the oxidation of NH_3 in pure cultures makes the statement that favorable influence of a more perfect aeration (in liquid cultures) is very marked.

In 1903 Boullanger and Massol (9) incubated their cultures in Erlenmeyer flasks containing slag (scoires cassés on petits morceaux) broken into small pieces, thus increasing the surface of their liquid cultures. They shook the flasks three or four times every day. Rapid nitrification was observed.

Muntz and Laine (28) worked out an arrangement based on the same principle as above with the exception that they used bone black and peat instead of "scoires" of Boullanger and

Massol with the result that they obtained intensified nitrification.

Chr. Barthel (1) claimed that by his aeration method a strong nitrification took place.

The use of large bottomed shallow vessels has found favor with many investigators. Lönneis and Green (23) obtained maximum oxidation when the ratio between the depth and surface of the layer of culture medium was 1 to 90.

Meyerhof (27) very considerably accelerated nitrification by the simple means of passing air thru his culture media. Bonazzi (6) obtained greater intensity in nitrification by exposing larger surface and by constantly changing the surface exposed to air. He does not, however, think that increased availability of air to the culture had anything to do with the increase in nitrification. Boullanger (10) followed the work he had done with Massol in 1903. This time he used peat and carried out the experiment on a larger scale to see if production of nitrates by biological process could be put on a commercial basis. He let a solution of $(\text{NH}_4)_2\text{SO}_4$ flow thru several cubic meters of peat inoculated with the nitrifying organisms. The amount of liquid passed thru the peat daily was 200 liters containing 7.5 grams $(\text{NH}_4)_2\text{SO}_4$ per liter. He obtained in the solution at the exhaust 138.2 grams of calcium nitrate per liter.

In the preliminary work of isolation of the nitrifying organisms which has been described in detail (Part I) it was found that in the oxidation of 25 c.c. of nutrient solution

containing 0.1 percent $(\text{NH}_4)_2\text{SO}_4$ it took at the least 9 to 10 days even tho the inoculum was 1 c.c. To increase the rate of oxidation the following experiment was carried out.

Experimental

Pint bottles were fitted with double holed rubber stoppers thru which passed glass tubes bent at right angles, one of them reaching the bottom of the bottle. They were sterilized and into each of these was put 200 c.c. of sterile solution #11 which contains:

	gms.
NaNO_2	1.0
Na_2CO_3	1.0
K_2HPO_4	0.5
NaCl	0.5
MgSO_4	0.3
$\text{Fe}_2(\text{SO}_4)_3$	trace
Water (conductivity)...	1000 c.c.

These solutions were inoculated with a culture of nitrate former which had gone thru a number of subcultures. The sterile moist air was drawn thru the solution.

The determination of nitrates was made by the phenol-di-sulphonic acid method and the hydrogen ion concentration by the "color standards" of Medalia (25). Advanced information of

the material published later (26) was also made use of.

OXIDATION OF NaNO_2 by NITRATE FORMER.

Table I.

Date			1	2	3
April 26	Original NaNO_2	Mgs.	200	200	200
-	pH		8.5	8.5	8.5
	NaNO_2 as NO_3	Mgs.	0	0	0
April 28	pH		8.4	8.4	8.4
	NaNO_2 as NO_3	Mgs.	52.4	65.5	78.5
May 1	pH		8.5	8.6	8.6
	NaNO_2 as NO_3	Mgs.	186.1	177.1	128.5
May 3	pH		8.6	8.6	8.6
	NaNO_2 as NO_3	Mgs.	200	200	200

Table II.

Date			1	2	3
May 6	Original NaNO_2	Mgs.	200	200	200
May 7	pH		8.8	8.8	8.8
	NaNO_2 as NO_3	Mgs.	20.3	20.3	20.3
May 9	pH		8.8	8.8	8.8
	NaNO_2 as NO_3	Mgs.	32.7	33.9	55.2
May 11	pH		8.6	8.6	8.6
	NaNO_2 as NO_3	Mgs.	157.1	151.8	151.8

As will be seen from Table I for the complete oxidation of 200 c.c of nitrate solution, it took one week by this method of aeration, whereas without aeration it usually took more than one week to oxidize 25 c.c. of the same medium. The rate of oxidation with aeration was 28.6 Mgs. NaNO_2 per day.

The experiment was repeated with the results recorded in Table 2. Oxidation up to the period the experiment was continued took place at the maximum rate of 31.4 Mgs. of NaNO_2 per day. .

Oxidation of $(\text{NH}_4)_2\text{SO}_4$.

The apparatus used for the oxidation of $(\text{NH}_4)_2\text{SO}_4$ was exactly similar to the one used for the oxidation of NaNO_2 . Solution #1 containing the following nutrients was used:

	Gms.
$(\text{NH}_4)_2\text{SO}_4$	1.0
K_2HPO_4	1.0
NaCl	2.0
MgSO_4	0.5
$\text{Fe}_2(\text{SO}_4)_3$	trace
Water (conductivity) ..	1000 c.c.

200 c.c. of the sterile solution was added to the sterile pint bottles and inoculated with a liquid culture of nitrite former and moist sterile air drawn thru the solution.

The nitrites were determined by colorimetric method using sulphanilic acid and alpha - naphthylamin hydrochloride

in acetic acid.

OXIDATION OF $(\text{NH}_4)_2\text{SO}_4$ BY NITRITE FORMER IN A
MEDIUM NOT SUPPLIED WITH BASE.

Table III.

Date		1	2	3
Original	$(\text{NH}_4)_2\text{SO}_4$ Mgs.	200	200	200
April 26	pH	7.1	7.1	7.1
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	0	0	0
April 28	pH	7.1	7.0	7.0
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	0	8.98	2.8
May 1	pH	7.1	6.6	6.6
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	2.6	21.3	trace
May 3	pH	6.9	6.4	6.9
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	8.4	22.0	trace
May 5	pH	6.8	6.4	6.8
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	17.2	24.3	trace
May 6	pH	6.6	6.5	6.9
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	29.4	26.0	trace
May 7	pH	6.5	6.4	6.9
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	51.8	26.0	2.3
May 9	pH	6.2	6.4	6.5
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	36.0	26.0	4.3
May 13	pH	6.2	-	-
	$(\text{NH}_4)_2\text{SO}_4$ as NO_2 (Mgs.)	44.1	-	-

The results reported in Table III were at first difficult to understand. The activity of the organism as indicated by the production of NO_2 was very slow. For the first five days only a trace of nitrite was observed. Then the amount of nitrite increased more rapidly in two of the cultures, while culture #3 did not show much activity until the end.

- The explanation was to be found in the reaction of the medium which was determined every time the solution was tested for nitrite. The solution was about neutral at the start and as the activity of the organism increased the acidity of the solution increased. Since there was no base supplied the acid could not be neutralized.

The results indicated that even the neutral solution does not support much activity and whatever oxidation occurred was very slow.

In the following experiment ^{with} ammonia oxidation, the solution was supplied with an excess of MgCO_3 to react with the acid produced.

OXIDATION OF $(\text{NH}_4)_2\text{SO}_4$ BY NITRITE FORMER IN
PRESENCE OF BASE MgCO_3 .

Table IV.

						KH_2PO_4		KH_2HPO_4	
		1	Check no inoc- ulum		5	check no inocu- lum	6	check no inocu- lum	
$(\text{NH}_4)_2\text{SO}_4$	Mgs. May	200	200	June	200	200	200	200	
$(\text{NH}_4)_2\text{SO}_4$ as NO_2	Mgs. 28	0	0	7	0	0	0	0	
pH		8.1	8.1		8.1	8.1	8.1	8.1	
$(\text{NH}_4)_2\text{SO}_4$ as NO_2	June	200	0	June	105	0	101.2	0	
pH	9	8.0	8.1	15	7.3	8.1	7.4	8.1	

As will be seen from Table IV, culture #1 oxidized all of the ammonium sulphate in 11 days. Its rate of oxidation was about 18 Mgs. per day. In cultures 5 and 6 mono- and di-basic phosphates respectively were used. Evidently there was not much difference between the two forms of phosphorus.

MgCO_3 as a base in the oxidation of $(\text{NH}_4)_2\text{SO}_4$ in solution cultures of nitrite formers was quite without any of the injurious effects as observed by Löhnis and Green (6) or Gaarder and Hagemm (15) who tried both MgCO_3 and CaCO_3 as buffer for their media. The results reported there are in agreement with the recommendation and usage of Winogradsky (44.)

Reaction of Media.

Table III clearly indicates that ammonia oxidation takes place very slowly or not at all at pH 7. From the results of Table IV it is plain that at pH 8 intense nitrification takes place. Gaarder and Hagem (15) have shown from their experiments that optimum for nitrite production is at pH 7.9. Meyerhof (27) on the other hand obtained the maximum nitrite formation at pH 8.4.

Very rapid oxidation of NO_2 takes place according to the results in Table I at the reaction of pH 8.5 to 8.8. This result is conformed by the work of Meyerhof (27) who finds the optimum reaction for nitrate former between pH 8.5 and 9.3. Gaarder and Hagem (15) obtained maximum oxidation of nitrite at pH 6.8 to 7.3.

To find out if it is possible to further accelerate the rate of oxidation of NH_3 by the nitrifying organisms, the apparatus described below was devised. It was prepared so that it should include the benefit derived from aeration together with the advantage accruing from constantly changing the surface of liquid exposed to the attack of the organisms.

A long tube 2.25 inches in diameter and 5 feet 2 inches in length was almost filled with pieces of marble of the size of peas. One end was closed by rubber stopper S, with two holes. Thru one of these holes passed a tube A bent at right angles and projected about 2 inches into the tube. Thru the other

hole passed a straight glass tube B which did not project inside the tube any farther than the rubber stopper. At the other end of the tube a wad of glass wool W covering a length of three inches was packed moderately tightly on the column of marble. This end was closed with a rubber stopper with two holes thru which passed two glass tubes. There was a space of one inch left between the glass wool and the rubber stopper. One of the tubes E thru the stopper projected $3/4$ of an inch into this space and directly below it on the glass wool was placed a watch glass C.

Another tube 6 feet long and 2.25 inches wide was fitted similarly except that instead of marble, clean washed split pea-size limestone was used.

Both the tubes A and B were wrapped in coarse linen and sterilized by passing thru steam intermittently.

The tubes were set up vertically, the glass wool ends being at the top. The straight tube at the lower end was connected to a sterile flask F provided with a siphon arrangement. The bent tube was attached to a train of sterile water, sterile cotton and sterile sulphuric acid thru which air was drawn. At the upper end tube E was connected to a siphon from a sterile ammonium sulphate nutrient solution #1. The other tube G was connected to the suction pump.

To establish a flora of nitrite formers on the marble

in A and on the limestone in B, 190 c.c. portions of two vigorously oxidizing organisms were allowed to drip slowly thru E.

Preliminary Experiment.

A fresh nutrient solution #1 of ammonium sulphate was then allowed to drip slowly thru E and the suction pump started to draw air thru the tubes. The rate of dripping was regulated to four large drops every 3 minutes.

Samples of the solution collected at the bottom in the Erlenmeyer flasks were taken at different periods and analyzed for nitrite. The Tables 5 and 6 show the rate of oxidation of ammonium sulphate per liter of solution dripped during different intervals. The maximum rate of oxidation at the given rate of flow was observed on July 9th between 11 A.M. and 6 P.M. when 266 mgs. of ammonium sulphate per liter were oxidized.

This oxidation of 266 Mgs. of ammonium sulphate was caused partly by the organisms in the tubes and partly also by the organisms washed into the flask where the liquid had been accumulating for four days.

DRIPPING TUBE A

OXIDATION OF $(\text{NH}_4)_2\text{SO}_4$ IN LONG TUBES CONTAINING MARBLE.

Table V.

	Date & time samples taken	Inter- val hours	Rate of flow	Reaction of solution collected at bottom pH	Mgs. (Total) ($\text{NH}_4)_2\text{SO}_4$ found as NO_2 in liter
a	July 5	18	4 drops	6.6.	439.0
b	" 7	48	in 3 minutes	6.6	613.5
c	" 9 11 A.M.	48	"	6.6	746.0
d	July 9 6 P.M.	7	"	7.0	1012.0

DRIPPING TUBE B

OXIDATION OF $(\text{NH}_4)_2\text{SO}_4$ IN LONG TUBES CONTAINING LIMESTONE.

Table VI.

	Date & time samples taken	Inter- val hours	Rate of flow	Reaction of solution collected at bottom pH	Total Mgs. ($\text{NH}_4)_2\text{SO}_4$ oxidized to NO_2 per liter
a	July 5	18	4 drops	6.6	232.4
b	July 7	48	in 3 minutes	6.6	381.65
c	July 9 11 A.M.	48	"	6.6	506.0
d	July 9 6 P.M.	7	"	7.0	716.0

To determine the exact amount of ammonium sulphate oxidized per day, the following experiment was conducted in which the outflow liquid was collected and removed at definite intervals. The amount of liquid was measured, analyzed and the nitrite content calculated. By doing this the nutrient solution was exposed mainly to the oxidizing action of the organisms in the tubes, and not to those that collected in the receiving flask F.

OXIDATION OF $(\text{NH}_4)_2\text{SO}_4$ IN A LONG TUBE CONTAINING MARBLE.

Table VII.

Tube A Marble pieces.

Date	Period run Hrs.Min.	Rate of flow per Min.	Reaction in Receiver	Amount of liquid in receiving c.c.	$(\text{NH}_4)_2\text{SO}_4$ oxidized in period run Mgs.	$(\text{NH}_4)_2\text{SO}_4$ oxidized per 24 hours. Mgs.	Equivalent to Mgs. $(\text{NH}_4)_2\text{SO}_4$ oxidized 100 c.c.
1 July 23	5 30	5	6.8	125.0	19.6	104.6	15.92
2 July 31	4 0	7	7.3	175.0	53.78	202.0	19.31
3 July 31 & Aug. 1	11 0	4.5	7.4	270.0	75.71	165.4	29.09
4 Aug. 1	8 0	3	7.3	136.0	2.04	48.1	3.58

OXIDATION OF $(\text{NH}_4)_2\text{SO}_4$ IN A LONG TUBE CONTAINING LIMESTONE.

Table VIII.

Tube B Limestone.

Date	Period run hrs.min.	Rate of flow drops per min.	Reaction in Receiver pH	Amount of liquid in receiv- er c.c.	$(\text{NH}_4)_2\text{SO}_4$ oxidized in period run Mgs.	$(\text{NH}_4)_2\text{SO}_4$ oxidized per 24 hours Mgs.	Equivalent to Mgs. $(\text{NH}_4)_2\text{SO}_4$ oxidized in 100 c.c.
1 July 23	5 30	5	6.7	230	32.96	175.8	14.3
2 July 31	4 0	7	7.5	160	22.75	136.5	14.2
3 July 31 to Aug. 1	11 0	9.5	7.7	480	139.60	304.5	29.0
4 Aug. 1	8 0	3	7.5	150	106.00	318.0	17.1

As seen from Table VII maximum rate of oxidation in tube containing marble was 202 mgs. per day. In the tube containing limestone the organism attained a maximum rate of oxidation of 318 mgs. per day. This was assuming that the rate of oxidation throughout the rest of the day remained as high as it was during the 8 hours the liquids were collected. This assumption, however, is not warranted because of the rate of oxidation is quite irregular. This has been noticed by Bonazzi (8), Winogradsky (44) found that after 20 days nitrification in one of his cultures remained station-

ary instead of showing progressive increase, as it had done previously.

An external physical cause of washing down the organisms by the liquid dripping down might also have had some influence in decreasing the rate of oxidation on August 1st noticed in the tube with the marble.

The high rate of oxidation obtained in these experiments is a result of optimum aeration, removal of products of oxidation by the downward flow of liquid and exposition of a large surface of liquid to the bacterial activity. Where aeration was practiced as in the case of milk bottle experiments reported at the beginning less than 20 mgs. of ammonium sulphate was oxidized per day. However, comparison between these two experiments and much less between two different investigators, unless experiments are conducted on identically similar conditions of apparatus, inoculum, aeration, etc., is liable to mislead.

Conclusions

1. When a current of air was passed thru a liquid culture of nitrate former in pint milk bottles, 31.4 mgs. of sodium nitrite was oxidized per day.
2. Under the same conditions 18 mgs. of ammonium sulphate was oxidized by nitrite former.
3. When a nutrient solution of ammonium sulphate was

allowed to drip into the top of a long tube containing limestone of the size of split peas on the surface of which a flora of nitrite formers had been established and a current of air was drawn from the bottom, the rate of oxidation of ammonium sulphate reached the maximum of 318 mgs. per day.

4. Vigorous oxidation of ammonium sulphate by nitrite former took place when the reaction of the liquid medium was around pH 8.0; for nitrate former the optimum reaction was between pH 8.5 and 8.8.

5. Magnesium carbonate used as a base in the oxidation of ammonium sulphate was without any injurious toxic effect.

A C K N O W L E D G M E N T

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PART III.

NITRATES AND NITRIFICATION IN FIELD SOILS.

Russel's remark that "the nitrate supply in soil is very commonly a limiting factor in crop production in Great Britain, so that any process which increases the nitrate supply tends to increase productiveness, and vice versa" holds good for conditions in Iowa.

The conditions best suited for the production of nitrates in soil are optimum moisture, plenty of easily decomposable organic matter, sweet and well aerated soil with good tilth and the presence of available phosphorus and potassium. These conditions are also among the main requirements for good crop yields. Temperature affects both nitrification and crop yield alike.

This would indicate that where there is large production of nitrates there should also be found large crop yield.

HISTORICAL

The subject of nitrification in soil has been the theme of considerable investigation in the last thirty years and a complete review of the literature would be impossible in this paper. Among the more important contributions the following may be mentioned.

Effect of Manure on Nitrification.

In 1892 Warington (29) stated that the greatest nitrification was observed at Rothamstead in plots which had received farm yard manure. According to Velbel (27), the first result of the application of manure was to produce a certain amount of denitrification, but later in summer nitrification was more rapid in manured than in unmanured soil and the favorable effect on soil was still noticeable after 4 years. Brown (5) found that the nitrifying power of the soil tested was increased by applying manure up to 16 tons per acre while 20 tons of manure caused a depression in nitrifying power. Jensen (15) in 1911 observed a slightly higher accumulation of nitrate on manured fallow land than on fallow check. In 1912 he noticed that dry yard manure decreased the nitrifying activity in the soil. Composted manure produced a slightly higher nitrifying activity than did the dry yard manure but both showed slightly less nitrates than the checks. "At all times" according to White (32) "stable manure mixture showed more nitrates than the soil alone". In his investigations on nitrification of stable manure nitrogen in cultivated soil, Barthel (3) found that the amount of nitrogen formed from the ammonia nitrogen of the manure was constant and independent of the amounts of manure added. Fraps (9) carried on nitrification experiments with 500 gram portions of soil in percolators extracting the nitrates at intervals of 4 weeks. It was found that the addition of manure to the soil resulted in a decrease in the amount of nitrates in the percolators. "There is no rela-

tion" he writes "between the nitrifying capacity of the soil and the nitrification of the manure". Whiting and Schoonover (33) concluded that stable manure is efficient in nitrate production, especially when used with phosphate and limestone. Vogel (28) observed that nitrification was greatly reduced by the addition of straw.

Effect of Lime on Nitrification.

The addition of alkali salts with superphosphate but without nitrogenous manure gave, according to Warrington (29) a distinct increase in the amount of nitrate found in field soil. Withers (31) concluded that the addition of lime or carbonate of lime from time to time hastens nitrification. Bacteriological studies of field soils conducted by Brown (4) in Iowa showed that three ton applications of lime gave proportionately greater increases in the nitrifying power of the soil than two ton applications.

Waste lime on fallow land was observed by Jensen (15) to cause strong nitrate accumulation, being more effective in this regard than any other treatment. Calcium carbonate additions markedly increased the nitrification of all five soils used by Noyes and Conner (19) in their pot culture work. According to Vogel (28), heavy applications of calcium carbonate did not appreciably affect nitrification.

Effect of Phosphates on Nitrification

According to Warrington (29) Withers (31) Whiting and Schoonover (33) Fraps (9) Noyes and Conner (19) and Ames and Richmond (2) nitrification was stimulated by addition of phosphate fertilizers. Markenna (18) however, observed that addition of phosphate in many cases diminished the rate of nitrification.

Effect of Moisture on Nitrification

Deherain and Demoussy (7) stated that for optimum bacterial activities in soils 17 percent of moisture was necessary. Nitrification required higher moisture content in light soils than in heavy soils. The optimum moisture content for nitrification was determined by Sharp (22) to be at 19 percent. According to Greaves and Stewart and Hirst (11) the greatest quantity of nitrate nitrogen was found where the amount of water applied was 15 inches. Sievers (24) found that nitrification took place very slowly in Palouse silt loam soil when the moisture content was below 15 percent. Hutchinson and Milligan (13) fixed three-eighths saturation as optimum for nitrification; Gainey and Metzger (10) fixed it at two-thirds saturation. According to Noyes and Conner (19) 50 percent saturation is optimum for nitrification. Greaves and Carter (12) found that it was between 50 and 60 percent saturation.

Effect of Season on Nitrification.

King and Whitson (16) Pongel and Guirand (20) Jensen (14)(15) Russel (21) Albrecht (1) and Whiting and Schoonover (33) are unanimous in the conclusion that the greatest amount of nitrification takes place in soil in late spring and early summer, the amount of nitrate diminishing in summer. Some of the above investigators have observed that in early autumn there is a slight increase in nitrate content. Vogel (28) observed the highest nitrification in October and November, and he also noted that the treatment of the soil had less effect upon nitrification than the time of year.

On the other hand experiments on nitrification in soils indicate to Limmerman and Wichers (17) that hitherto advanced proofs of a direct periodic influence of the time of the year on the life activities of soil organisms independent of temperature and other physical weathering influence are insufficient.

Effect of Crops on Nitrates in Soil.

That the period of loss in nitrate content of soil in summer time coincides with the period of rapid growth of crops was noticed by King and Whitson (16) Stewart and Greaves (26) Russel (21) Albrecht (1) and Whiting and Schoonover (33). According to Brown (5) there is close relationship between bacterial

activities and the crop producing power of soils. Vogel's (28) experiments with potatoes and barley in the soils indicated that the productiveness was in direct relation to the nitrifying capacity. Burgess (6) considers nitrification by far the most accurate biological soil test for predicting the probable fertility of Hawaiian soils. But Jensen (15) thinks that the differences in the average seasonal accumulations of nitrates could not have been due entirely to the differences in beet crop yields on the plots he worked with.

The object of these experiments was to determine the effect of treatment, season and moisture on the nitrate content and nitrifying power of soil and to determine if these bear any relation to crop yields.

EXPERIMENTAL

This investigation was conducted on the plots in Series 1300 on the Agronomy Farm of the Iowa Agricultural Experiment Station. This series consists of 36 plots, 0.1 acre in size, 1.7 rods wide by 9.5 rods long; the division strips are 6.5 feet wide and a cropped border 7 feet wide runs around the 36 plots. The topography is almost uniform except in two places covered by plots 18, 19 and 30, 31 and 32 where there are low spots. Most of the plots are on the Webster silty clay loam except for a few that are in part or wholly on Webster loam, Carrington loam, Webster silt loam or Clarion loam as shown in the accompanying figure 1.

TABLE I
Treatment of Plots

No.	Treatment
1300	Check
1301	1600# Manure
1302	400# Manure (annually)
1303	1600# Manure plus lime
1304	2400# Manure plus lime
1305	Check
1306	3200# Manure plus lime
1307	4000# Manure plus lime
1308	1600# Manure plus 200# R.R.P. plus lime
1309*	1600# Manure plus 200# lime plus 80# bone meal.
1310*	Check
1311	1600# Manure plus 20# acid phosphate (ann.) plus lime
1312	1600# Manure plus 200# R.R.P. plus 20# KCl plus lime
1313	1600# Manure plus 30# 2-8-2 (ann.) plus lime
1314	1600# Manure plus 30# 2-12-5 (ann.) plus lime
1315	Check
1316*	30# 2-8-2 hill application
1317*	30# 2-8-2 broad cast
1318*	30# 2-8-2 plus lime broadcast
1319*	Lime
1320*	Check
1321*	Burnt lime
1322*	1600# manure plus lime
1323*	1600# Manure plus burnt lime
1324	Crop residues
1325	Check
1326	Crop residues plus lime
1327*	Crop residues plus burnt lime
1328	Crop residues plus 200# R.R.P. plus lime
1329*	Crop residues plus limestone plus bone meal
1330*	Check
1331	Crop residues plus 20# acid phosphate (ann.) plus lime
1332	Crop residues plus 30# 2-8-2 (ann) plus lime
1333	Crop residues plus 200# R.R.P. plus 20# KCl. plus lime
1334	Crop residues plus 30# 2-12-5 (ann.) plus lime
1335	Check

The treatments indicated in Table 1 are applied once in four years unless otherwise stated. A four year rotation of corn, corn, oats and clover is practiced. This series of plots is in the second rotation. In 1921 when this investigation was carried on it was planted to corn. In 1920 it was in clover. On the crop residue plots 1324 to 1334 (inclusive) the second cutting of clover was turned under and from the rest of the plots in the series all clover was removed. The plots marked with a star (*) were not used in this investigation.

Method of Taking Soil Samples

In a plot measuring 0.1 of an acre there is undoubtedly great variation in samples taken at different spots on the same day. Waynick (30) holds that "a limited number of samples, as 10 or 16, are subject to wide variations and can only be interpreted as having a low degree of accuracy". Russel (21) thinks that "a fair amount of uniformity exists in the nitrate content of a plot which has been uniformly treated and the differences between the various mixed samples do not generally amount to more than about 2 parts per million".

In this work 8 samples were taken in all from June to October. Each sampling was made from a different part of the plot. The loose surface soil was removed from an area 2 feet square and with a spatula sterilized with 50 percent alcohol the soil to a depth of $6 \frac{2}{3}$ inches was mixed thoroughly and a sample

TABLE II
Moisture Determinations

	Percent Moisture on							
Plot	June	July	Aug.:	Aug.:	Sept.:	Sept.:	Oct.:	Average
No.	21	25	9	30	20 *	23	12	
1300	17.5	7.5	6.5	12.5	20.0	17.5	16.0	13.9
1301	17.25	7.5	7.5	14.0	20.0	17.5	15.0	14.1
1302	15.0	13.5	6.5	13.0	20.0	17.5	18.0	14.7
1303	15.5	10.0	6.0	15.0	20.0	17.5	18.0	14.6
1304	17.0	9.0	7.5	12.5	20.0	17.5	18.0	14.5
1305	16.0	13.5	9.0	16.5	22.5	17.5	18.0	16.1
1306	14.5	12.5	10.0	16.5	22.5	20.0	21.0	16.7
1307	17.5	12.5	10.0	17.5	22.5	22.5	23.5	18.0
1308	17.5	15.0	14.0	17.5	20.0	22.5	20.0	18.1
1311	19.0	15.0	14.0	19.0	25.0	22.5	26.0	20.1
1312	19.0	15.0	16.5	17.5	20.0	20.0	25.0	19.0
1313	22.5	16.5	14.0	17.5	25.0	20.0	26.0	21.2
1314	22.25	18.5	17.5	21.0	25.0	27.5	27.5	22.8
1315	24.0	20.0	15.0	20.0	26.0	25.0	27.5	22.5
1324	17.5	12.5	11.0	15.0	20.5	20.0	20.0	16.5
1325				19.0	16.0	20.0	21.5	19.1
1326	14.0	11.5	13.0	19.0	20.0	17.5	26.0	17.3
1328	20.0	10.5	10.5	19.0	20.0	22.5	23.0	17.9
1331	19.5	16.5	12.5	18.5	25.0	25.0	20.0	19.5
1332	23.75	19.0	15.0	16.0	31.5	27.5	23.0	22.3
1333	21.25	19.0	11.5	17.0	25.0	25.0	18.0	18.1
1334	17.5	11.0	6.0	10.0	25.0	25.0	17.0	15.9
1335	15.5	10.0	5.0	9.0	25.0	20.0	13.0	13.9
Ave.	18.3	12.5	10.8	16.2	22.5	21.2	21.0	

* Rained the previous day

TABLE III

Pounds Per Acre of Nitrate Nitrogen in the Surface 6 2/3 Inches
of Soil in Corn Plots Variously Located as Determined
at Several Depths in 1921

Plot : No. :	Treatment	:June : 21	:July : 25	:Aug. : 9	:Aug. : 30	:Sept. : 20	:Sept. : 23	:Oct. : 12	:Ave. :
1300	Check	77.62	56.04	22.84	16.18	11.78	10.42	15.24	30.02
1301	1600 M (annual)	68.74	49.22	25.68	23.26	12.20	22.04	17.40	31.22
1302	400 M (annual)	67.52	81.30	24.98	12.84	8.18	7.76	16.34	31.28
1303	1600 M.L.	55.66	39.82	22.56	14.70	14.70	7.42	16.34	23.52
1304	2400 M.L.	90.50	45.88	21.88	14.98	11.50	10.76	22.94	34.06
1305	Check	52.48	31.62	14.00	12.46	7.54	7.46	11.94	19.64
1306	3200 M.L.	46.88	36.58	15.02	19.74	8.06	6.78	18.74	21.74
1307	4000 M.L.	64.04	34.84	16.00	19.78	11.68	12.00	17.78	25.14
1308	1600 M. 200 R.P.L.	45.82	42.26	20.66	22.58	7.96	8.04	24.00	24.48
1311	1600 M. 20A.P.(ann.) L.	49.02	29.74	16.66	8.50	11.21	10.80	12.44	19.78
1312	1600 M. 200 R.P. 20 K.L	41.98	48.92	18.78	11.06	8.06	12.20	17.34	22.65
1313	1600 M. 30(2-8-2)(Ann.)L.	64.88	57.98	22.98	26.86	10.34	22.72	13.40	31.30
1314	1600 M. 30(2-12-5) An.L.	95.70	32.68	20.78	16.30	11.74	12.40	22.34	30.26
1315	Check	68.90	71.00	26.44	21.50	11.86	15.90	21.52	33.88
1324	Cr.	32.78	22.22	13.12	11.60	8.00	11.10	15.76	16.38
1325	Check				25.58	6.82	12.98	13.76	14.78
1326	Cr. L.	50.56	46.10	13.04	18.48	7.28	11.98	18.66	23.72
1328	Cr. 200 R.P.L.	74.10	38.08	11.26	11.26	8.86	8.48	12.78	23.60
1331	Cr. 20 A.P.(an) L.	49.04	36.78	18.48	13.48	9.44	6.58	11.50	20.76
1332	Cr. 30 (2-8-2)(ann.) L.	63.18	19.36	20.04	7.72	10.52	7.88	12.20	20.12
1333	Cr. 200 R.P. 20 K.L.	51.66	27.16	11.58	9.14	7.62	8.20	10.98	18.04
1334	Cr. 30 (2-12-5)(ann.) L.	57.62	36.76	10.32	8.54	6.80	11.84	14.20	20.98
1335	Check	79.90	40.34	9.52	7.40	11.60	10.88	12.20	24.54
	Average	61.28	44.02	16.98	16.88	10.32	12.28	17.42	

of about a kilogram was taken in a sterile jar and brought to the laboratory.

Moisture determinations were made on ten gram samples and the results are shown in Table 2.

Nitrate Determinations.

100 gram samples were shaken with 300 c.c. water and the amounts of nitrates in the solution were determined by the phenoldisulphonic acid method. Aluminium hydroxide was used to classify the solutions. The nitrate content was calculated on dry basis.

Two other 100 gram samples of each soil were placed in tumblers, 100 mgs. $(\text{NH}_4)_2\text{SO}_4$ added, moisture brought up to optimum (50 percent saturation) covered with a tin cover and incubated for 4 weeks. Loss of moisture was made up every week. After 4 weeks the nitrate content was determined. This is used to indicate the power of soil to nitrify $(\text{NH}_4)_2\text{SO}_4$.

Effect of Season on Nitrate Content.

Table 3 shows very markedly the effect of season on the nitrate content of the soil. The highest amount of nitrate in almost all the soils was found in the sampling made in June. There was a gradual decrease in nitrates in the next month and from then on the decrease was very rapid till the middle of September when the nitrates reached a minimum. From then on

in the next three weeks there was a decided rise in the amount of nitrates in all the plots.

These observations are in conformity with those made by other investigators noted previously. The small increase in nitrates during the fall was noted also by Whiting and Schoonover (33).

Effect of Moisture Content.

No definite conclusions can be drawn from this data regarding the effect of moisture on the soil nitrate but general observations may be made. From a broad viewpoint it seems that 18 to 19 percent was the optimum moisture content for nitrification in these field soils. The average moisture content for the whole field shows that on June 21 when the soil contained the maximum nitrate nitrogen the average moisture content of the entire 36 plots was little over 18 percent. Plots 1307, 1308, 1326 and 1328 which showed on the average for the whole season a high nitrate content had an average moisture content of about 18 percent during the same period. But the plots 1301, 1302 and 1304 which also showed considerable nitrates had only 14 to 15 percent moisture on the average during the entire period. However, in general the majority of the plots that showed maximum nitrate production had a moisture content of about 18 percent.

The results obtained on August 9 are significant. The rapid decrease in the nitrate content at this period

TABLE IV

Plots	Nitrate Nitrogen lbs. per acre in Soil	Progressive Calculation between checks	Increase for treatment		Nitrifying power ex- pressed as lbs. per acre	Progressive Calculation between checks
			lbs.	percent		
1300	30.02				426.0	
1301	31.22	19.64	11.58	58.95	408.0	292.8
1302	31.28	19.64	11.64	59.27	408.0	292.8
1303	23.52	19.64	3.88	19.73	351.4	292.8
1304	34.06	19.64	14.42	72.41	393.8	292.8
1305	19.64				292.8	
1306	21.74	21.06	0.68	3.23	341.0	309.1
1307	25.14	22.48	2.66	12.00	379.8	325.6
1308	21.48	23.90	0.58	2.43	385.4	341.9
1311	19.78	28.17	-8.39	-29.80	481.6	392.1
1312	22.65	29.59	-6.94	-23.44	456.0	407.5
1313	31.30	31.01	0.29	1.21	485.6	423.8
1314	30.26	32.43	-2.17	-6.69	466.6	440.2
1315	33.88	33.86			456.6	456.0
1324	16.38	16.69	-0.31	-1.87	300.6	258.0
1325	14.78	14.78			236.1	236.1
1326	23.72	15.76	7.96	50.56	306.1	247.0
1328	23.60	17.71	5.89	26.40	366.6	258.0
1331	20.76	20.64	0.12	0.58	440.2	268.9
1332	20.12	21.61	-1.49	-6.88	456.2	274.3
1333	18.04	22.58	-4.54	-20.11	421.8	279.9
1334	20.98	23.56	-2.58	-10.95	304.4	285.3
1335	24.54	25.54			290.8	29.8

IV

Recessive mutation phen types	Increase for treatment		Corn yield 1921 lbs. per acre	Yield Calculated from check	Increase for treatment	
	lbs.	percent			lbs.	percent
			4700			
.8	115.2	39.37	5000	4700	300	6.383
.8	115.2	39.37	4800	4700	100	2.127
.8	58.6	20.02	4800	4700	100	2.127
.8	101.0	34.50	5400	4700	700	14.89
			4700			
.1	32.82	10.62	5600	4610	990	21.47
.6	54.24	16.65	6100	4520	1580	34.96
.9	43.46	12.72	5400	4430	970	41.90
.1	90.52	23.15	5600	4160	1440	34.61
.5	48.54	11.91	5500	4070	1430	35.14
.8	61.76	14.57	5600	3980	1520	38.19
.2	26.38	5.992	6000	3890	2110	53.99
.0			3800			
.0	42.6	16.51	4190	3932	258	6.531
.1			3920			
.0	59.06	24.46	4080	3961	119	3.004
.0	108.62	42.10	4430	4043	387	9.572
.9	171.28	63.69	4590	4166	424	10.181
.3	181.87	66.30	4460	4207	253	6.015
.9	141.94	50.66	4490	4248	242	5.695
.3	19.07	6.68	4640	4289	351	8.164
.8			4330			

coincided with the decrease in the moisture content which averaged 11 percent for the whole series. This would indicate that the influence of the time of the year on nitrification is a result mainly of the seasonal conditions particularly high or low rainfall. Plot 1335 which thruout the season had in general a higher amount of nitrate than the adjacent plots 1334 and 1333 showed lesser amounts on the 9th and 30th of August when its moisture content was also lower. A comparison of the nitrates in the soils on September 20 and 23 is of interest. There was a heavy rainfall on the 19th and its effect is shown in the smaller amounts of nitrates on the 20th.

Effect of Manure on Soil Nitrates.

In Table 4 the increase in nitrate content with the various treatments is expressed in percent. The method of progressive increase between two check plots was used to calculate the effect due to the treatment. In the case of plots 1301 to 1304, however, only 1305 was used as a check discarding plot 1300 because of the residual effect of large amounts of manure this plot had received before it was used for experimental work.

The addition of 8 tons of manure once in four years (plot 1301) or in 4 annual applications (plot 1302) gave about 60 percent increase in nitrate content. The 12 ton application (plot 1304) showed a still greater increase in nitrates. Applications of manure larger than 12 tons did not show as large increases.

TABLE V

The Nitrification of $(\text{NH}_4)_2\text{SO}_4$ Calculated as Pounds Per Acre
of Nitrate Nitrogen in the Surface 6 2/3 Inches of Soil
in Corn Plots Variously Treated

Plots:	Treatment	: : June : 21	: : July : 12	: : July : 25	: : Aug. : 9	: : Aug. : 20	: : Sept. : 20	: : Sept. : 23	: : Oct. : 12	: : Ave.
1300	Check	532.4	443.6	276.2	426.8	351.6	763.6	299.2	315.2	426.0
1301	1600 M	514.8	474.4	286.0	498.8	332.6	441.2	401.8	314.8	408.0
1302	400 M (ann.)	380.4	454.8	344.8	414.0	374.0	569.6	398.8	337.8	408.0
1303	1600 M.L.	406.2	456.2	330.8	440.4	364.2	288.0	314.8	210.0	351.4
1304	2400 M.L.	575.4	454.0	328.0	456.6	297.0	531.0	341.6	167.4	393.8
1305	Check	427.2	405.0	296.0	329.6	321.9	226.6	204.8	131.4	292.8
1306	3200 M.L.	504.6	438.2	318.0	404.0	333.4	326.0	359.8	134.4	341.0
1307	4000 M.L.	491.6	554.0	291.4	325.4	293.0	609.2	281.6	192.2	379.8
1308	1600 M. 200 R.P.L.	513.4	430.4	293.0	489.8	360.0	467.6	259.8	269.4	385.4
1311	1600 M.L. 20 AP(ann.) L.	578.6	465.4	370.6	489.8	231.0	824.0	399.0	294.8	481.6
1312	1600 M.200R.P. 20 K.L.	472.6	484.2	313.4	504.4	408.0	756.8	365.8	343.0	456.0
1313	1600 M.30(2-8-2) an. L.	607.0	523.6	352.0	498.0	352.6	844.4	392.8	313.8	485.6
1314	1600 M.30(2-12-5)an.L.	566.4	493.6	279.8	421.0	383.4	872.8	414.2	301.0	466.6
1315	Check	697.2	512.4	290.2	448.2	398.0	794.6	375.4	132.6	456.0
1324	Cr.	336.0	444.4	218.0	379.6	309.4	277.8	252.6	187.6	300.6
1325	Check					274.0	311.8	233.0	125.6	236.1
1326	Cr. L.	395.6	335.6	219.6	379.4	294.8	400.0	305.8	118.0	306.1
1328	Cr. 200 R.P.,L.	384.6	394.6	233.2	442.4	370.4	455.8	225.8	326.8	366.6
1331	Cr.20 A.P.(an).L.	534.4	502.0	306.0	377.2	310.2	826.6	375.4	290.2	440.2
1332	Cr.30(2-8-2) an.L	608.2	505.8	262.0	487.0	289.2	858.4	390.4	249.4	456.2
1333	Cr.200 R.P.20K.L.	528.8	434.0	260.0	404.0	265.0	923.2	390.2	168.8	421.8
1334	Cr. 30(2-12-5) an.L.	406.4	483.8	230.0	398.8	318.4	276.4	251.4	170.0	304.9
1335	Check	384.8	395.4	193.2	476.8	154.0	311.6	281.8	129.2	290.8
Ave.		496.6	453.8	286.0	432.0	329.8	562.6	326.0	227.0	

The results on plot 1303 receiving 8 tons of manure cannot be explained with the data at hand. Evidently some unknown factor had an effect there.

The year previous to this investigation the plots 1324 to 1336 inclusive were in clover and the second cutting was turned under in the fall. Plot 1326 which received lime beside the clover turned under showed 50 percent increase in nitrates over the check plot 1325. In plot 1326 which received clover but no lime, nitrification was depressed. Probably the acids produced by the decomposing green manure were not neutralized and therefore had an adverse effect on the nitrifying organisms.

Among the mineral fertilizer treatments rock phosphate (plots 1308 and 1328) gave the largest increase in nitrate nitrogen.

Nitrifying Power of Soil.

Table 5 shows the nitrifying power of the soils or their ability to serve as a medium for the growth of nitrifying organisms (Fraps 9). The extent of the growth of nitrifying organisms is measured by the amount of $(\text{NH}_4)_2\text{SO}_4$ oxidized in a given period under optimum conditions of moisture and temperature.

In general it is apparent that all the plots receiving treatments showed a higher nitrifying power than the untreated plots. The manure treatments up to 12 tons gave large increases in nitrifying power. 16 and 20 ton applications showed

small increases. The plots receiving crop residues together with mineral fertilizers showed the maximum nitrifying power. The plots receiving acid phosphate were higher in nitrifying power than those receiving rock phosphate. The more even distribution of the acid phosphate in the soil may account for greater bacterial growth, but probably the solubility of the phosphorus is the more important factor.

Effect of Treatment on Crop Yields.

Up to and including 20 tons the larger the amount of manure applied the greater was the crop yield obtained in 1921. The 20 ton application gave a 35 percent increase and the 16 ton application gave 21.5 percent increase while 12 and 8 ton applications gave 14.9 and 6.4 percent increases respectively. It would appear from this that the 20 ton application was the most efficient.

In the manure plots rock phosphate with potassium gave as large an increase in crop yield as did acid phosphate. In the crop residue plots rock phosphate alone gave as large an increase as acid phosphate.

Commercial fertilizers applied with manure gave the largest increases in crop yield of all treatments. With crop residues, however, the effect of commercial fertilizers was not marked.

Crop residues alone in the case of plot 1324 gave almost as great an increase in crop yield as plots 1332 and 1334 which received commercial fertilizers along with crop residues.

Nitrate Content, Nitrifying Power and
Crop Yield

Figure 2 shows graphically the percentage increase or decrease in nitrate content, nitrifying power, and the crop yield (1921) induced by the treatments. The figures for nitrates and nitrifying power represent averages of the results obtained from 7 samplings.

The figure indicates from the curves for plots 1301, 1302, 1304, 1326 and 1328 that the crop yield is proportional to nitrate content. The curves for plots 1306, 1307, 1308, 1311, 1312 and 1314 apparently contradict the above statement but in reality they do not. The large increase in crop yield has drawn heavily on the nitrate content and hence the low nitrate content obtained.

The nitrate content of a soil is proportional to the nitrifying power of that soil. This is indicated clearly in the curves for plots 1301, 1302, 1304, 1306, 1307, 1308, 1326 and 1328. The apparent discrepancy between plots 1311 and 1312 is explained as before by the factor of high crop yield and in plots 1331 and 1332 the high nitrifying power and low nitrate content is explained by the high moisture content during the period of

sampling which has leached some of the nitrates, also the crop yield on these plots is fairly high over the check plot.

Summary

1. In all the plots examined there was a large accumulation of nitrates in June with a gradual decrease in July, and a rapid decrease in August and September when it reached the minimum. In October there was a slight increase in nitrates.

2. The optimum moisture content for nitrification in this field soil was about 18 percent.

3. The application of manure up to 12 tons per acre gave the greatest increase in nitrate accumulation and nitrifying power over the untreated soil. 16 and 20 ton applications gave smaller increases than those secured when 12 tons per acre were added.

4. The largest application of manure, 20 tons per acre, gave the greatest increase in crop yield in 1921, being 35 percent over the untreated plot, the 16 ton application gave 21 percent, 12 tons gave 15 percent and 8 tons gave 6.4 percent increase over the untreated plot.

5. The crop residues turned under the previous fall increased the nitrate content of the soil when lime was present. The increase in nitrifying power due to the crop residues was also large. Crop residues increased the crop yield 6.5 percent over the check.

6. Rock phosphate showed greater accumulation of nitrates in soil than did either acid phosphate or commercial fertilizers. Rock phosphate with crop residue gave about as large increase in crop yield as did acid phosphate.

7. Acid phosphate increased the nitrifying power of soil more than the rock phosphate did.

8. Complete commercial fertilizers along with crop residues did not give any greater increase in crop yield than did the crop residue alone.

9. The crop yield on a soil bears a direct relation to the nitrate content and the nitrifying power of that soil.

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